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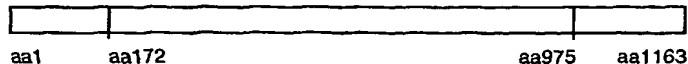
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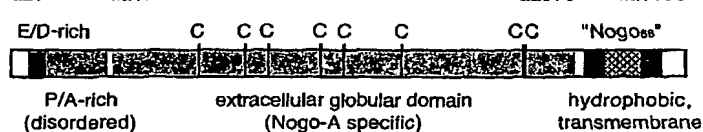
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- (71) Applicant (for all designated States except US): **PIERIS PROTEOLAB AG** [DE/DE]; Lise-Meitner-Strasse 30, 85354 Freising-Weihenstephan (DE).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **SKERRA, Arne** [DE/DE]; Max-Lehner-Strasse 18, 85354 Freising (DE). **FIEDLER, Markus** [DE/DE]; Schillerstr. 6, 06114 Halle/Saale (DE).
- (74) Agent: **VIERING, JENTSCHURA & PARTNER**; Steinsdorfstrasse 6, 80538 München (DE).
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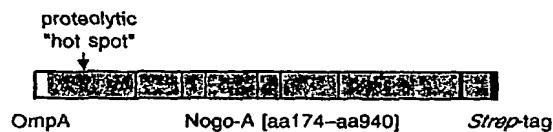
Nogo-A (NI-220) cDNA:



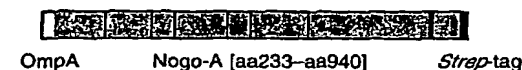
Protein characteristics:



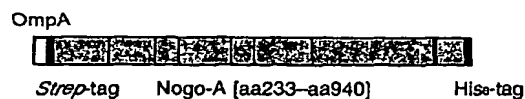
Soluble rec. fragment NI-Fr1



Soluble rec. fragment NI-Fr2



Soluble rec. fragment NI-Fr4



(57) Abstract: The present invention refers to an isolated truncated Nogo-A polypeptide that corresponds to a truncated form of the Nogo-A protein consisting of the amino acids 174 to 940 of the full length protein of rat Nogo-A or of the amino acids 246 to 966 of the human full length Nogo-A protein.

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SOLUBLE TRUNCATED POLYPEPTIDES OF THE NOGO-A PROTEIN

5 The present invention relates to soluble truncated polypeptides of the Nogo-A protein, nucleic acid molecules encoding such polypeptides as well as to methods for the production of such polypeptides. The present invention also relates to methods for identifying and generating compounds having detectable affinity to a Nogo-A protein, in particular such compounds that have a neutralizing effect on the neurite-growth-inhibiting  
10 activity of Nogo-A. Therefore, the present invention is also directed to the use of compounds having binding affinity and preferably also a neutralizing effect on the neurite-growth-inhibiting activity of Nogo-A as diagnostics or pharmaceuticals.

The very limited capacity of the adult central nervous system (CNS) for axonal  
15 regeneration is a phenomenon of broad and ongoing scientific as well as medical interest (see, e.g., Horner and Gage, (2000) *Nature*, **407**, 963-970). In contrast, sprouting and elongation of lesioned axons readily occurs in the peripheral nervous system (PNS). Inhibitory effects and non-permissible properties of CNS tissue, in particular of CNS myelin and oligodendrocytes, probably contribute considerably to the restriction of  
20 neuronal regeneration and plasticity. *In vitro*, CNS myelin and oligodendrocyte membranes induce growth cone collapse (Bandtlow et al., (1990) *J. Neurosci.*, **10**, 3837-3848).

Based on earlier observations of the inhibitory effect of CNS myelin on neurite outgrowth (Caroni and Schwab, *J. Cell Biol.*, (1988) **106**, 1281-1288) the myelin-associated neurite  
25 growth inhibitor NI-220 (Spillmann et al., (1998) *J. Biol. Chem.*, **273**, 19283-1929), later called Nogo-A (Huber and Schwab, *Biol. Chem.*, **381**, 407-419), was identified in bovine spinal cord tissue as a predominant protein of oligodendrocytes that prevents axonal growth. The corresponding cDNAs from rat and man were recently described (Chen et al., (2000) *Nature*, **403**, 434-439; GrandPré, et al., (2000) *Nature*, **403**, 439-444; Prinjha et al.,  
30 (2000) *Nature*, **403**, 383-384). The *nogo* gene encodes three distinct proteins, Nogo-A, Nogo-B, and Nogo-C, which apparently arise by alternative splicing and/or promoter usage. Of those only the full length Nogo-A transcript is specifically expressed in oligodendrocytes and hence made mainly responsible for their neuronal growth inhibitory activity (Spillmann et al., supra; Chen et al., supra).

35 In addition, a monoclonal antibody named IN-1 is known (Caroni and Schwab, (1988) *Neuron*, **1**, 85-96; European Patent Application 0 396 719). This antibody was shown to

neutralize the inhibitory activity of Nogo *in vitro* (Bandtlow et al., (1990) *J. Neurosci.*, **10**, 3837-3848; Spillmann et al., supra) and *in vivo*, giving rise to long-distance regeneration and improved plastic changes of injured CNS fiber tracts (Schnell and Schwab, (1990) *Nature*, **343**, 269-272; Z'Graggen et al., (1998) *J. Neurosci.*, **18**, 4744-4757).

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The variable domain cDNAs of the antibody IN-1 were cloned from the hybridoma cell line, followed by the bacterial production of the corresponding recombinant murine Fab fragment, whose functionality was demonstrated *in vitro* (Bandtlow et al., (1996) *Eur. J. Biochem.*, **241**, 468-475). A partially humanized IN-1 Fab fragment was produced by *E. coli* fermentation and shown to successfully promote regeneration of corticospinal axons in adult rats after spinal cord lesion *in vivo* (Broesamle et al., (2000) *J. Neurosci.*, **20**, 8061-8068). The recombinant IN-1 Fab fragment also induced significant elongation of injured cochlear fibres upon intrathecal treatment (Tatagiba et al., (2002) *Acta Neurochir. (Wien)*, **144**, 181-187) and a pronounced sprouting response of Purkinje cells after injection into the intact adult cerebellum (Buffo et al., (2000) *J. Neurosci.*, **20**, 2275-2286).

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However, two problems exist for studying axonal growth and for developing methods for promoting neuronal regeneration in the CNS.

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First, as a membrane-bound protein Nogo-A is traditionally isolated only in small amounts and in a laborious procedure from CNS myelin. The heterologous production of the full length 1163 Nogo-A protein (1163 residues in case of the rat Nogo-A, 1192 residues in case of the human protein) in mammalian cells (Chen et al., supra; GrandPré, et al., supra, is apparently also not suitable for providing the rather large amounts of pure protein which are, for example, needed to study the inhibitory activity of Nogo at the molecular level (e.g. by X-ray crystallography) or in screening assays for compounds with neutralizing activity. According to Chen et al., supra enrichment of recombinant Nogo by means of affinity chromatography yielded a protein extract from CHO cells in which Nogo represented only about 1 to 5 % of the protein present.

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In addition, Prinjha et al., supra describe the production of a soluble fusion protein of human Nogo-A in which amino acid residues 1 to 1024 are fused to a human Fc polypeptide. Furthermore, GrandPré et al., supra, describe the expression of a 66-residue lumenal/extracellular fragment of human Nogo (amino acids 1055 to 1120 of human Nogo-A) as fusion protein with glutathione S-transferase (GST). Both fusion proteins are reported to be a potent neurite-growth inhibitor. However, no further use of these fusion

proteins in investigating the inhibitory effect of Nogo or in the development of potential pharmacological treatments have been described.

Second, the only molecule for which a notable neutralizing effect on the neurite-growth-inhibiting activity of Nogo-A has been observed is the antibody IN-1. However, both the original monoclonal antibody IN-1 as well as its bacterially produced F<sub>ab</sub> fragment have a rather low affinity for the antigen Nogo-A. Due to this low affinity, and in case of the monoclonal IgM antibody also due to its large size, the antibody IN-1 do not represent a well-suited candidate for practical applications, in particular for therapeutic purposes.

Therefore, there is still a demand for an assay system with which, a) regeneration processes can be investigated at the molecular level, and b) molecules having improved binding affinity to Nogo-A, and optionally also with improved neutralizing effect on the neurite-growth-inhibiting activity of Nogo-A, can be found.

Accordingly, it is an object of the invention to overcome the limitations of the prior art and to provide a system that meets the above needs.

This object is solved, among others, by the polypeptides and the method having the features of the independent claims.

Such a polypeptide is an isolated truncated Nogo-A polypeptide that corresponds to a truncated form of the Nogo-A protein consisting of the amino acids 174 to 940 of the full length protein of rat Nogo-A (SEQ ID NO: 1, 1163 amino acids) or of the amino acids 246 to 966 of the human full length protein (SEQ ID NO: 2, 1192 amino acids).

The inventors have found that such a N- and C-terminally truncated form of the Nogo-A protein has many advantages. First, it can be produced as a soluble, stable protein, that does not undergo significant proteolytic degradation, without using a fusion protein that confers solubility. Second, this polypeptide can be produced in amounts that are sufficient, for example, for large scale screening assays or crystallization experiments. Third, the truncated soluble protein maintains the neurite-growth-inhibiting activity of the full length protein. This is in so far surprising as the so-called "Nogo-66" region comprising the amino acid residues 1055 to 1120 of human Nogo-A, that belong to that C-terminal part of the full length protein that is deleted in the fragments of the present invention, was recently reported to be a potent nerve cone collapsing factor, i.e. a potent inhibitor of the axonal regeneration (GrandPré, et al., supra). Consequently, the good stability and availability of

the inventive truncated Nogo-A protein together with its inhibitory activity render it to be an excellent target that can be used in the screening for molecules having neutralizing activity.

5 For reasons of clarity it is noted that the numbering of the amino acid residues, when referring to the rat protein, is used in accordance with the numbering of the 1163 residues containing full length protein of rat described by Chen et al, supra (SEQ ID NO: 1, EMBL data base accession code: AJ242961). When referring to the human protein, the residue numbering is used in accordance with the sequence of the full length human protein (SEQ  
10 ID NO:2, EMBL data base accession number AJ251383; 1192 residues) described by GrandPré, et al., supra and Prinjha et al., supra, (cf. Fig.6 where the amino acid sequences as deposited as also shown). It is noted in this respect, that the present results indicate that the truncated fragments of Nogo-A according to the present invention are derived from one  
15 exon of the gene.

In a preferred embodiment, the polypeptide of the invention corresponds to the truncated form of the Nogo-A protein which consists of the amino acids 223 to 940 of the full length protein of rat Nogo-A. In a further embodiment, this truncated polypeptide corresponds to the Nogo-A protein that consists of the amino acids 270 to 900 of the full length protein of  
20 rat Nogo-A. Generally speaking, a preferred truncated polypeptide of the invention corresponds to a truncated Nogo-A protein of rat that comprises at least the sequences positions 323 to 890 in order to be able to include all cysteine residues that are present at positions 323, 403, 443, 536, 676, 885 and 890 in the wild-type rat protein.

25 In a further preferred embodiment, the polypeptide corresponds to a truncated form of the Nogo-A protein that consists of the amino acids 334 to 966 of the full length human Nogo-A protein. Preferably, the truncated form of the Nogo-A protein consists of the amino acids 380 or 424 to 699 or 850 of the full length human Nogo-A protein. In an alternative embodiment, the truncated Nogo-A polypeptide corresponds to a truncated  
30 human Nogo-A protein that comprises at least the sequences positions 424, 464, 559, 596, 699 and 912 which are occupied by cysteine residues in the human wild-type protein.

In general the truncated Nogo-A protein is not limited to a specific lower size but every truncated form falling within the boundaries defined by the amino acid positions 174 to  
35 940 of the full length protein of rat Nogo-A (SEQ ID NO: 1, 1163 amino acids) or 246 to 966 of the human full length protein, respectively, are in the scope of the invention as long as they have similar or the same inhibitory activity as the respective Nogo-A wild type

protein and/or preferably fold into a polypeptide having a three-dimensional structure similar or identical to the wild type protein. Accordingly, truncated Nogo-A forms having a length of (only) e.g. 19, 20, 25, 50, 100, 150 or 200, 250 or 300 residues are also comprised in the invention if they yield a functional active Nogo-A peptide or protein. The  
5 functionality can be assessed in a common neurite outgrowth assay as described here or e.g. by Chen et al., supra, or by GrandPré et al., supra. In one aspect, fragments are preferred which include all cysteine residues that seem to play a role in the folding of the protein. In case of the Nogo-A protein of rat, such a fragment includes the sequence corresponding to positions 323 to 890 of the full length Nogo-A sequence. In the case of  
10 the human protein, such a fragment includes the amino acid residues 424 to 699 or 424 to 890 (cf. above).

The truncated form of the Nogo-A protein of the invention can be derived from the natural sequence of any suitable mammal and non-mammal species. Although the truncated  
15 polypeptide is preferably of mammalian origin, for instance of human, porcine, murine, bovine or rat origin, the use of Nogo orthologues from invertebrates or lower species such as *Drosophila melanogaster* or *Caenorhabditis elegans* is also within the scope of the invention. In one preferred embodiment the mutein is a truncated variant of Nogo-A protein of human or rat origin.

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In preferred embodiments the polypeptide of the present invention is selected from the group consisting of:

- a) the polypeptide having the amino acid sequence consisting of amino acid residues 174 to 940 of the full length rat Nogo-A protein (SEQ ID NO: 1);
- 25 b) the polypeptide having the amino acid sequence consisting of amino acid residues 233 to 940 of the full length rat Nogo-A protein (SEQ ID NO: 1);
- c) the polypeptide having the amino acid sequence consisting of amino acid residues 246 to 966 of the full length human Nogo-A protein (SEQ ID NO: 2);
- d) the polypeptide having the amino acid sequence consisting of the amino acid  
30 residues 334 to 966 of the full length human Nogo-A protein (SEQ ID NO: 2);
- e) a polypeptide having at least 50 % sequence identity to any of the polypeptides a) to d) wherein the fragment of human Nogo-A consisting of amino acids 1 to 1024 is excluded;
- f) a fragment of any of the polypeptides a) to e), wherein the fragment consisting of  
35 amino acids 624 to 639 of the full length rat Nogo-A protein is excluded.

As stated above, such a fragment of a truncated Nogo-A protein can contain not more than 19, 20, 50, 100, 150, 200, 250 or 300 amino acid residues.

5 The term "sequence identity" or "identity" as used in the present invention means the percentage of pair-wise identical residues – following homology alignment of a sequence of a polypeptide of the present invention with a sequence in question - with respect to the number of residues in the longer of these two sequences.

10 Thus taking, for example, as polypeptide in question the polypeptide that is used in Chen et al., supra for the generation of the antiserum "AS Bruna" and that consists of the amino acid residues 762 to 1163 (i.e. 402 residues) of the full length rat protein, the identity as defined in the present invention is calculated as follows. Compared to the fragment of the invention consisting of amino acids 174 to 940 of the rat Nogo-A, this "AS Bruna" polypeptide shares (following homology alignment) 940-762 = 179 pair-wise identical  
15 residues with the inventive polypeptide. Since the polypeptide of the invention is the longer of the two fragments (767 residues), the identity is calculated to be  $179/767 = 0.233$  or 23.3 %. As a second example, the identity of this "AS bruna" polypeptide with a inventive fragment consisting of amino acid residues 233 to 890 of the rat full length Nogo-A is as follows. The "AS Bruna" polypeptide shares 890-762= 129 identical residue  
20 with the polypeptide of the invention. Again the polypeptide of the invention is the longer fragment (890-233=658 residues). The identity is thus  $129/658 = 0.196$  or 19,6 %.

25 In a further preferred embodiment the truncated human Nogo-A polypeptide of the invention begins with an amino acid residue selected from the amino acids 246 to 424 and ends at a residue selected from amino acids 912 to 966 of the full length protein. A preferred truncated polypeptide of the rat Nogo-A protein begins with an amino acid residue selected from the amino acids 174 to 233 and ends at a residue selected from amino acids 890 to 940 of the full length Nogo-A.

30 In accordance with the above definition of the term "identity", the polypeptide of the invention can have the natural amino acid sequence of Nogo-A throughout the truncated form. On the other hand, the truncated polypeptide disclosed here can also contain amino acid mutations compared to the wild-type protein as long as those mutations a) do not yield a protein with less than 50 % sequence identity and preferably b) yield a protein that folds  
35 into a three-dimensional structure identical or comparable to that of one of the truncated forms of Nogo-A of the present invention and/or has the same biological neurite growth inhibitory activity. This also means, that a polypeptide having a sequence identity of equal

to or greater than 50 % is also considered to fall within the scope of the present invention, even if it does not have any neurite growth inhibitory activity at all but a different biological activity.

5 The differences in the amino acid sequence can be caused, for example, by mutations, substitutions, deletions, insertion (of continuous stretches) of amino acid residues as well as by N- and/or C-terminal additions introduced into the natural amino acid sequence of the truncated Nogo-A forms, i.e. the truncated Nogo-A consisting of amino acid residues 174 to 940 of the full length rat Nogo-A protein (SEQ ID NO: 1) or amino acid residues  
10 246 to 966 of the full length human Nogo-A protein (SEQ ID NO:2) or a smaller fragment thereof as disclosed herein.

Such modifications of the amino acid sequence within or outside these boundaries of the selected protein include directed mutagenesis of single amino acid positions, for example,  
15 in order to simplify the subcloning of the Nogo gene or its parts by incorporating cleavage sites for certain restriction enzymes. Furthermore, mutations can be introduced within the truncated polypeptide in order to improve certain characteristics of the chosen Nogo-A protein, for example its folding stability or folding efficiency or its resistance to proteases. For example, if recombinant production is to take place in an oxidizing thiol/disulfide  
20 redox milieu *in vivo* or if the protein is to be used in an oxidizing environment, cysteine residues can be replaced by serine or alanine in order to avoid processes such as dimerization or oxidation of the thiol group which deteriorate the folding efficiency or the life-time of the purified protein when stored. Therefore, the cysteine residues that are not crucial for the folding of the protein can be replaced in the Nogo-A variants of the present  
25 invention. In one embodiment of fragments which are based on Nogo-A of rat origin, at least one of the cysteine residues at positions 403, 536, 574 and 676 are substituted by a suitable amino acid (cf. Examples).

In preferred embodiments, the polypeptide of the invention has at least 60, 70, 72, 75, 80,  
30 85, or 90 or 95 % sequence identity to the truncated form of the Nogo-A protein described here. In accordance with the meaning of the term "identity", the substitution of an amino acid with a chemically similar amino acid is considered to be a conservative substitution that maintains the identity. Examples of such conservative substitutions are the substitution for one another: 1) alanine, serine, threonine; 2) aspartic acid and glutamic acid; 3)  
35 asparagine and glutamine; 4) arginine and lysine; 5) isoleucine, leucine, methionine, valin; and 6) phenylalanine, tyrosine, tryptophan.



Although the Nogo-A protein of the present invention comprises a stable soluble monomeric polypeptide chain which can be produced as such, it is also possible to produce the truncated Nogo-A protein as fusion protein. The fusion partner can be connected to the N- and/or the C-terminus of the Nogo-A polypeptide and is preferably a protein, a protein domain or a peptide. In case of a peptide, this peptide is preferably an affinity tag such as the Strep-Tag® or the Strep-tag® II (Schmidt et al., J. Mol. Biol. 255 (1996), 753-766) or an oligohistidine, e.g. penta- or hexahistidine tag.

For the heterologous production, a peptide such as a signal sequence and/or an affinity tag is operably fused to the N- terminus or to the C- terminus of the Nogo-A protein. Affinity tags such as the Strep-Tag® or the Strep-tag® II (Schmidt et al., supra) or oligohistidine tags (e.g., His<sub>5</sub>- or His<sub>6</sub>-tags) or proteins such as glutathione-S-transferase which can be used for purification by affinity chromatography and/or for detection (e.g. using the specific affinity of the Strep-tag® for streptavidin) are examples of preferred fusion partners. Further examples of fusion partners which can be advantageous in practice are binding domains such as the albumin-binding domain of protein G, the immunoglobulin-binding domains of protein A or oligomerizing domains, if, for example, an avidity effect is desired. As indicated, the term fusion protein as used herein also includes truncated Nogo-A polypeptides that are equipped with a signal sequence. Signal sequences at the N-terminus of a polypeptide according to the invention can be suitable to direct the polypeptide to a specific cell compartment during its biosynthesis, for example into the periplasm of *E. coli* or to the lumen of the endoplasmic reticulum of the eukaryotic cell or into the medium surrounding the cell. In doing so, the signal sequence is usually cleaved by a signal peptidase. It is also possible to use other targeting or signalling sequences which may also be located at the N-terminus of the polypeptide and which allow the localization thereof in specific cell compartments. A preferred signal sequence for secretion into the periplasm of *E. coli* is the OmpA signal sequence. A large number of further signal sequences is known in the art.

Therefore, the present invention is also directed to a method for the production of a truncated Nogo-A polypeptide or a fusion protein thereof. In this method, the Nogo-A polypeptide or the fusion protein of the Nogo-A polypeptide is produced starting from the nucleic acid coding for the Nogo-A polypeptide either by means of an *in vitro* transcription and translation system (e.g. a cell free system) or by means of genetic engineering methods either in a bacterial or eukaryotic host organism. The polypeptide is then isolated from this *in vitro* system or from this host organism or its culture.

For this purpose a suitable host cell is usually first transformed with a vector comprising a nucleic acid molecule encoding, for instance, the truncated human Nogo-A consisting of amino acid residues 334 to 966 of the invention. The host cell, which can be any prokaryotic or eukaryotic host cell is then cultured under conditions which allow the biosynthesis of the polypeptide (via transcription/translation of the nucleic acid or gene). The polypeptide is then usually recovered either from the cell or from the cultivation medium. Since the Nogo-A protein seems to contain structural disulfide bonds it is preferred to direct the polypeptide into a cell compartment having an oxidizing thiol/disulfide redox milieu by use of a suitable signal sequence. Such an oxidizing milieu is present in the periplasm of bacteria such as *E. coli* or in the lumen of the endoplasmic reticulum of a eukaryotic cell and usually favours the correct formation of the disulfide bonds. It is, however, also possible to produce a polypeptide of the invention in the cytosol of a host cell preferably *E. coli*. In this case the polypeptide can, for instance, be produced in form of inclusion bodies, followed by renaturation *in vitro*. A further option is the use of specifically mutated strains which have an oxidizing milieu in the cytosol and thus allow production of the native protein in the cytosol.

The invention is also related to a nucleic acid molecule encoding a truncated Nogo-A polypeptide according to the invention or a fusion protein thereof.

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In one preferred embodiment the nucleic acid molecule consists of or comprises the nucleotide sequence of positions 522 to 2822 of the coding sequence of rat Nogo-A (encoding the amino acids 174 to 940 of rat Nogo-A) deposited under accession number AJ242961 in the EMBL database or the nucleotide sequence of positions 699 to 2822 (encoding the amino acids 233 to 940 of rat Nogo-A) of this coding sequence. In another preferred embodiment the nucleic acid molecule consists of or comprises the nucleotide sequence of positions 738 to 2900 of the coding sequence of human Nogo-A (encoding the amino acids 246 to 966 of human Nogo-A) deposited under accession number AJ251383 in the EMBL data or of positions 1002 to 2900 of this coding sequence (encoding the amino acids 334 to 966 of human Nogo-A).

30

Since the degeneracy of the genetic code permits substitutions of certain codons by other codons which specify the same amino acid and hence give rise to the same protein, the invention is not limited to a specific nucleic acid molecule but includes all nucleic acid molecules comprising a nucleotide sequence coding for a truncated Nogo protein with an amino acid sequence according to the present invention.

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The nucleic acid molecule encoding a truncated Nogo-A polypeptide disclosed here can be operably linked to a regulatory sequence to allow expression of the nucleic acid molecule in a host cell (in vivo) or its transcription and translation in a cell-free system (in vitro).

5 A nucleic acid molecule such a DNA is regarded to be "capable of expressing a polypeptide" if it contains nucleotide sequences which contain transcriptional and translational information and if such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequences sought to be expressed are connected  
10 in such a way as to permit gene expression. The precise nature of the regulatory regions and elements needed for gene expression may vary from organism to organism, but shall, in general, include a promoter region which, in prokaryotes for example, contains both the promoter regulatory sequence that can comprise a transcriptional region functional in a cell and a transcriptional terminating region functional in a cell. Elements used for transcription  
15 or translation are promoters, operators, enhancers, leader sequences, transcription initiation sites and transcription termination sites, polyadenylation signals, ribosomal binding sites such the Shine-Dalgarno sequence and the like. The gene expression may also be inducible. These regulatory sequences and/or the truncated Nogo-A protein of the invention can be part of a vector. Accordingly, the invention also refers to a vector  
20 comprising a nucleic acid sequence coding for the truncated Nogo-A protein as disclosed here.

In a further aspect, the present invention refers to a method for identifying a compound having detectable affinity to a Nogo-A protein, comprising the steps of:

- 25 (a) contacting a truncated Nogo-A polypeptide or a fusion protein thereof as defined above with a compound of interest under conditions that allow formation of a complex between the truncated Nogo-A protein and said compound; and  
(b) detecting complex formation by means of a suitable signaling method.

30 In an alternative embodiment the method for identifying a compound having detectable affinity to a Nogo-A protein comprising the steps of:

- (a) contacting a truncated Nogo-A polypeptide or a fusion protein thereof as defined above with a plurality of compounds of interest under conditions that allow formation of a complex between the truncated Nogo-A protein and said compounds; and  
35 (b) enriching at least one compound of interest that has detectable binding affinity to the Nogo-A protein by screening or selection and/or isolating said at least one compound.

Thus, by use of the truncated Nogo-A proteins disclosed here, the invention provides for the first time a method which can be used in screening assays, e.g. using high throughput screening systems or evolutionary methods (combinatorial biology), for obtaining compounds having binding activity to a (wild-type) Nogo-A protein. For reason of clarity, it is noted that the term "a Nogo-A protein" is not restricted to a specific source but is to include Nogo-A proteins from mammalian and non-mammalian source, for example.

The term "plurality" as used herein means that at least two compounds that differ from each other in their structure, for example, in their amino acid or nucleotide sequences are present.

The method of identifying a compound having detectable affinity can be carried out with compounds (of interest) for which a binding affinity to Nogo-A has not been reported so far. However, the method of the invention can also be used for finding molecules starting from a (lead) compound which is known to bind a Nogo-A protein. Preferably the compound having detectable affinity is an organic molecule, a peptide, a polypeptide or a nucleic acid.

The term "organic molecule" preferably means an organic molecule comprising at least two carbon atoms, but not more than 7 rotatable carbon bonds having a molecular weight between 100 and 2000 Dalton, preferably 1000 Dalton and also a molecule including one or two metal atoms.

The signaling method used for detecting complex formation between the truncated Nogo-A protein and the binding compound may use every suitable signaling means which directly or indirectly generates in a chemical, enzymatic or physical reaction a detectable compound or a signal that can be used for detection. An example for a physical reaction is the emission of fluorescence after excitation with radiation or the emission of e.g.  $\alpha$ - or  $\beta$ -radiation by a radioactive label; alkaline phosphatase, horseradish peroxidase or  $\beta$ -galactosidase are examples of enzyme labels which catalyse the formation of chromogenic (colored), luminogenic or fluorogenic compounds which can then be used for detection. This signal can be caused by a label such as a fluorescent or chromogenic label which may be attached to one of the two binding partners, i.e. the truncated Nogo-A polypeptide or the compound of interest, or to a molecule that binds to either of the two binding partners. This signal can also be caused by the change of a physical properties which is caused by the binding, i.e. complex formation itself. An example of such a properties is surface plasmon

resonance the value of which is changed during binding of binding partners from which one is immobilized on a surface such as a gold.

Numerous formats for carrying out the method of identifying a compound having detectable affinity exist. A "colony screening" assay (Skerra et al., Anal. Biochem. 196 (1991), 151-155) can, for example, be used if the binding molecule is a polypeptide or peptide. The identification method can also be carried out as a solid phase assay, for example, in an ELISA format, in which the truncated Nogo-A polypeptide of the invention is immobilized in purified form in wells of an ELISA plate and is then brought into contact with the labeled molecule that is suspected to be able to bind to the Nogo-A protein. Such an assay format is more suitable, if binding activity is to be improved based on a compound with known but only weak binding activity. It is however also possible to label the truncated Nogo-A protein for detection of a possible complex formation.

Preferably, the compound having binding affinity to the Nogo-A protein also has a neutralizing effect on the neurite-growth-inhibiting activity of Nogo-A so that the compound may not only be used for diagnostic purposes (where pure binding without neutralizing effect can be sufficient, if tissue staining is desired, for example) but potentially also as pharmaceutical.

In case polypeptides or peptides with detectable binding affinity are to be found by use of the method of the invention, these peptides or polypeptides are preferably subjected to mutagenesis before contacting them with the Nogo-A protein in step a). This mutagenesis can either be a site-directed mutagenesis in which only one or a small number of amino acids are replaced by predetermined amino acids or a partially or entirely random mutagenesis, the latter leading to a library of protein or peptide mutants (muteins) (see Examples). Various strategies for mutagenesis are known to the skilled person in the field of combinatorial biology in order to create such a library.

If nucleic acids such as aptamers are employed as the compound of interest in the identification method of the present invention, they can of course also be employed in form of a library containing a large number of sequence variants. Likewise, also libraries of small organic molecules can be used in the method of identifying molecules having binding affinity to Nogo-A.

Examples of nucleic acids that can be used in a screening for a compound having binding activity to a Nogo-A protein are RNA- or DNA-molecules such as Spiegelmers® described

in WO 01/92655, for example. Spiegelmers® are mirror-image nucleic acids that are supposed to bind to and block a biological target with high affinity and specificity, comparable to an antibody.

5 If a proteinaceous molecule or a nucleic acid is to be identified as binding compound, the inventive method can comprise the step of enriching at least one mutant nucleic acid or mutein resulting from the mutagenesis and having detectable binding affinity to the Nogo-A protein by screening or selection and/or isolating said at least one mutein or mutant nucleic acid.

10 Preferred proteinaceous binding molecules that are used in a screening are chosen from the group consisting of antibodies or muteins based on a polypeptide of the lipocalin family. Examples of other proteinaceous binding molecules are the so-called glubodies described in the international patent application WO 96/23879, proteins based on the ankyrin  
15 scaffold (Hryniewicz-Jankowska, A. et al., (2002) *Folia Histochem. Cytobiol.* Vol. 40. 239-249) or crystalline scaffold (WO 01/04144, DE 199 32688) and the proteins described in Skerra (2000) *J. Mol. Recognit.* 13, 167-187.

20 An antibody may be used in any of the various forms of known (recombinant) fragments, e.g. as Fab fragment, single-chain F<sub>V</sub> fragment, F<sub>V</sub> fragment or diabody, all of which are well known to the person skilled in the art.

25 In a preferred embodiment of the identification method of the invention, the antibody mutant(s) used is (are) derived from the antibody IN-1 (cf. Examples). However, every antibody which is available in recombinant form or has been raised using the conventional immunization protocol of Köhler and Milstein (*Nature* 256 (1975), 495-497) can be tested for its binding properties. Also libraries, synthetic or from natural sources, which contain a large number of antibody muteins (usually more than approximately  $1 \cdot 10^7$  sequence variants) can be employed for the identification of molecules with detectable affinity to the  
30 Nogo-A protein. Such libraries are commercially available, for example, from Cambridge Antibody Technology, Cambridge, UK.

35 The lipocalin mutein is preferably an anticalin® as described in the German Offenlegungsschrift DE 197 42 706 or the international patent publication WO 99/16873; which is a polypeptide exhibiting specific binding characteristics for a given ligand, like antibodies (cf. also Beste et al., *Proc. Natl. Acad. Sci. USA*, 96 (1999) 1898-1903).

This lipocalin mutein is based on a member of the lipocalin family in which amino acid positions are mutated in the region of at least one of the four peptide loops, which are arranged at the open end of the cylindrical  $\beta$ -sheet structure. Preferably, these regions correspond (as described in WO 99/16873) to those segments in the linear polypeptide sequence comprising the amino acid positions 28 to 45, 58 to 69, 86 to 99 and 114) to 129 of the bilin-binding protein of *Pieris brassicae* or homologous positions in other lipocalins. Preferably amino acid positions in two, three or all four of these loops are mutated.

Suitable lipocalins that can be used as scaffold for the generation and identification of anticalins® with binding affinity to the Nogo-A protein are the bilin-binding protein (Bbp), the retinol-binding protein (Rbp), the apolipoprotein D (ApoD), the human neutrophil gelatinase-associated lipocalin (hNGAL), the rat  $\alpha_2$ -microglobulin-related protein (A2m) and the mouse 24p3/uterocalin (24p3). The use of human scaffolds such as hNGAL or ApoD is preferred for therapeutic applications.

An example of a binding molecule identified by the method of the invention as described here is the antibody fragment named II.1.8 which is derived from the antibody IN-1. The sequence of the variable domain of the light chain (VL) of the antibody fragment II.1.8 is shown as SEQ ID NO: 12. The sequence of the variable domain of the heavy chain (VH) of II.1.8 is identical to the sequence of IN-1 (Bandtlow et al, 1996, supra) and is shown in SEQ ID NO: 11. The antibody fragment II.1.8 shows improved affinity to the Nogo-A protein, thus allowing detection of Nogo-A in immunochemical experiments, for example.

For its use as diagnostic reagent the binding compound or molecule can be employed in a labeled form. In general, it is possible to label a binding compound such as the antibody fragment II.1.8 with any appropriate chemical substance or enzyme, which directly or indirectly generates in a chemical, enzymatic or physical reaction a detectable compound or a signal that can be used for detection. An example for a physical reaction is the emission of fluorescence after excitation with radiation or the emission of e.g.  $\alpha$ - or  $\beta$ -radiation by a radioactive label; alkaline phosphatase, horseradish peroxidase or  $\beta$ -galactosidase are examples of enzyme labels which catalyse the formation of chromogenic (colored), luminogenic or fluorogenic compounds which can then be used for detection. It is noted in this respect, that all of these labels discussed with respect to the (diagnostic) use of a binding compound can, of course, also be employed as signaling means in the method of identifying a binding compound of the invention.

The binding molecule can also be conjugated to a label such as an enzyme label, radioactive label, fluorescent label, chromogenic label, luminescent label, a hapten, biotin, digoxigenin, metal complexes, metals, and colloidal gold. Generally all labels which are used for antibodies, except those which are exclusively used in conjunction with the sugar moiety in the Fc part of immunoglobulins can also be used for conjugation to the muteins of the present invention. These conjugates can be prepared by methods known to the person skilled in the art. Alternatively, a proteinaceous binding compound identified by the method of the present invention can also be produced as chimera, for example, as fusion protein with an enzyme that catalyses a chromogenic or fluorogenic reaction (e.g. alkaline phosphatase, horseradish peroxidase, glutathione-S-transferase). Proteins with inherent chromogenic or fluorescent properties such as the green fluorescent protein (GFP) are suitable fusion partners, too.

The invention is further illustrated by the following examples and the attached drawings in which:

- Figure 1 shows recombinant Nogo-A fragments of the present invention;
- Figure 2 shows structural and functional characteristics of engineered IN-1 F<sub>ab</sub> fragments as examples for binding molecules obtained by the method of the invention for identifying a compound having detectable and improved affinity to a Nogo-A protein;
- Figure 3 shows an SDS PAGE of purified IN-1 F<sub>ab</sub> fragments as well as the antigen affinity determination for the wild-type IN-1 F<sub>ab</sub> fragment and its mutants by surface plasmon resonance (SPR);
- Figure 4 depicts the specific staining of myelin-rich regions in the rat brain using the IN-1 F<sub>ab</sub> fragment and its engineered mutants;
- Figure 5 shows the stepwise improvement of the biological activity of the IN-1 F<sub>ab</sub> fragment during affinity maturation as determined in an *in vitro* neurite outgrowth assay;
- Figure 6 shows the amino acid sequences of the full length Nogo-A protein of rat and human origin using the standard one letter code;



Figure 7 schematically depicts the expression vector pASK11-FR2.

Fig.1A schematically shows the structural characteristics of the native neurite growth inhibitor Nogo-A and of examples of recombinant soluble truncated fragments derived from it in the present invention. The fragment NI-Fr1 consists of the amino acids 174 to 940 of the full length Nogo-A rat protein with the Strep-Tag® fused to its C-terminus. The fragment NI-Fr2 consists of the amino acids 223 to 940 of the full length Nogo-A rat protein with the Strep-Tag® fused to its C-terminus. The fragment NI-Fr4 consists of amino acid 223 to 940 of the full length Nogo-A rat protein equipped with the Strep-Tag® at its N-terminus and a hexa-histidine-tag (His<sub>6</sub>) at its C-terminus. Fig.1B shows a SDS-PAGE analysis of the bacterially produced truncated fragment NI-Fr4. The periplasmic protein extract from *E. coli* JM83 harbouring pASK111-NIFr4 was loaded in lane 1. The flow-through of an IMAC column is shown in lane 2, eluted protein from IMAC column as applied to the streptavidin column in lane 3, flow-through of streptavidin column in lane 4, purified protein after streptavidin affinity chromatography in lane 5. Molecular sizes are indicated at the left. The proteins were visualized by staining with Coomassie Brilliant Blue.

Fig.2A shows the amino acid sequence of the VL domain (Kabat database accession no. 029919) of the monoclonal antibody IN-1 together with the substitutions introduced in the course of affinity maturation. Complementarity-determining regions (CDRs) are underlined according to the definition by Kabat et al. Sequences of proteins of immunological interest, 5th Ed. National Institutes of Health, Bethesda Md (1991), while amino acid positions are numbered consecutively. The mutations obtained by exchange of residues within CDR-L1 and CDR-L3 in the present invention are marked with bold letters below the wild-type sequence. Fig.2B shows a comparison of the antigen-binding activities of engineered Fab fragments in the ELISA experiments of Examples 5 and 6. Binding of the mutants I.2.6 (circles), II.1.8(squares) and I.2.6(<sup>L96</sup>V) (triangles) was compared with the binding of the wild-type IN1-Fab fragment (rhombs) to recombinant NI-FR2. The mutants I.2.6 and II.1.8 bind the truncated Nogo-A protein clearly in a concentration-dependent manner, whereas wild-type IN1-Fab fragment does not give rise to a significant binding signal.

Fig.3A shows an SDS/PAGE analysis of purified recombinant Fab fragments prepared according to the invention. Fab fragments were produced in *E. coli* JM83 harbouring the corresponding derivative of the vector pASK88 and purified by IMAC. Samples in the upper part were reduced with β-mercaptoethanol prior to SDS gel electrophoresis whereas

those in the lower part were kept unreduced: IN-1 (wild-type) F<sub>ab</sub> fragment is shown in lane 1, the Ala<sup>L32</sup>ØPhe mutant in lane 2, the I.2.6 mutant in lane 3; the I.2.6(L96V) mutant in lane 4; and the II.1.8 mutant in lane 5. Molecular sizes are indicated at the left. All F<sub>ab</sub> fragments appear as a homogeneous protein with stoichiometric presence of the light and heavy chains and show quantitative formation of their interchain disulphide bond. **Fig.3B** shows the measurement of the concentration-dependent interaction between the IN-1 F<sub>ab</sub> fragment (rhombs) and its optimized mutant II.1.8 (squares) with the recombinant Nogo-A fragment NI-Fr4 (immobilized on an Ni/NTA-sensor chip<sup>®</sup> at 285 to 305 ΔRU) by SPR (surface plasmon resonance) technique. Equilibrium values (differences in resonance units, ΔRU) determined after subtraction of the background signal in the absence of NI-Fr4 were plotted against the applied concentration of wild-type IN-1 F<sub>ab</sub> fragment or its II.1.8 mutant and finally fitted by non-linear regression.

**Fig.4** shows the specific staining of myelin-rich regions in the rat brain. The staining in **Fig.4A** was performed with an anti-MOG F<sub>ab</sub> fragment; the myelinated, MOG-positive *Corpus callosum* is marked by an asterisk and myelinated fibers of the *Capsula interna* in the *Corpus striatum* are indicated by arrows. **Fig.4B** shows staining with wild-type IN-1 F<sub>ab</sub> fragment, **Fig.4C** with I.2.6(L96V) F<sub>ab</sub> fragment, and **Fig.4D** with II.1.8 F<sub>ab</sub> fragment. **Fig.4E** shows staining with an anti-CD30 F<sub>ab</sub> fragment as negative control. Bound F<sub>ab</sub> fragment was detected in each case with a goat anti-human C<sub>κ</sub> antibody conjugated with alkaline phosphatase and revealed using the "Fast Red" procedure.

**Fig.5** depicts a graphical representation of the stepwise improvement of the biological activity of the IN-1 F<sub>ab</sub> fragment during affinity maturation. The columns show the mean neurite lengths of granula cells from the rat cerebellum cultured on a recombinant Nogo-A substrate – or just on poly-L-lysine as a control – whose inhibitory properties were neutralized in the presence of the IN-1 F<sub>ab</sub> fragment and its engineered mutants (applied at 100 μg/ml). Error bars correspond to standard deviations from triplicate experiments.

**Fig.6A** shows the amino acid sequence of the full length Nogo-A protein from rat described by Chen et al, supra. **Fig.6B** shows the amino acid sequence of the human full length Nogo-A protein described by GrandPré, et al., supra.

**Fig.7** shows a drawing of pASK111-NiFr2. This vector codes for a fusion protein made of the OmpA-signal sequence and the truncated Nogo-A fragment NI-Fr2 consisting of the amino acids 223 to 940 of the full length Nogo-A rat protein with the Strep-Tag<sup>®</sup> fused to its C-terminus (cf. **Fig.1a**). The entire structural gene is subject to the transcriptional

control of the tetracycline promoter/operator ( $tet^{p/o}$ ) and ends at the lipoprotein transcription terminator ( $t_{lpp}$ ). Further elements of the vector are the origin of replication ( $ori$ ), the intergenic region of the filamentous bacteriophage f1 (f1-IG), the chloramphenicol resistance gene ( $cat$ ) coding for chloramphenicol acetyl transferase and the tetracycline repressor gene ( $tetR$ ). A relevant segment from the nucleic acid sequence of pASK111-NiFr2 is reproduced together with the encoded amino acid sequence in the sequence protocol as SEQ ID NO: 13. The segment begins with the *Xba*I-restriction site and ends with the *Hind*III restriction site. The vector elements – with the exception of the  $cat$  gene - outside this region are identical with the vector pASK75, the complete nucleotide sequence of which is given in the German patent publication DE 44 17 598 A1.

### Examples

#### Example 1: Vector construction for Nogo fragments

Unless otherwise indicated, genetic engineering methods known to the person skilled in the art were used, as for example described in Sambrook et al.(supra).

A 2.3 kbp Nogo-A gene fragment was amplified from the cloned rat cDNA (Chen et al., supra) via PCR with the primers 5'-GCT CAG CGG CCG AGA CCC TTT TTG CTC TTC CTp(S)G-3' (SEQ ID NO: 3)(the *Eag*I restriction site is underlined) and 5'-GCT TTT AAC TAT GCT GCC CAT TTC TGp(S)T-3' (SEQ ID NO: 4). The single PCR product was digested with *Eag*I, purified from a 1 % agarose gel, and inserted into the multiple cloning region of pASK111 (Vogt and Skerra, *J. Mol. Recognit.*, 14,(2001) 79-86), which had been cut with *Bsa*I (resulting in a sticky end compatible with *Eag*I) as well as *Eco*47III, yielding pASK111-NiFr1. In this vector the Nogo-A fragment is precisely fused at its N-terminus (i.e. in front of residue 174) to the OmpA signal peptide. This vector leads to the production of a mature protein with a molecular mass of 85.0 kDa, including the *Strep*-tag at the C-terminus, after processing of the OmpA signal peptide fused in frame to the N-terminus. The vector pASK111-NiFr2 was constructed from pASK111-NiFr1 (SEQ ID NO: 14) by precisely deleting the N-terminal 59 codons from the cloned Nogo-A gene fragment via site-directed mutagenesis using the oligodeoxynucleotide 5'-GGT ATC CAT GTT CTT TAA AAG AGG CCT GCG CTA CGG TAG C-3' SEQ ID NO: (SEQ ID N NO: 5). Cys residues were replaced by Ser via site-directed mutagenesis with single-stranded DNA prepared from pASK111-NiFr2 using appropriate oligodeoxynucleotide primers.

The C-terminal *Strep*-tag encoded on pASK111-NiFr2 was exchanged by a His<sub>6</sub> affinity tag by site-directed mutagenesis with the oligodeoxynucleotide 5'-CAC TTC ACA GGT CAA GCT TAT TAA TGG TGA TGG TGA TGG TGA GCG CTT TTA ACT ATG CTG CCC-3' (SEQ ID NO: 6). A *KasI* restriction site was concomitantly introduced at the 5'-end of the cloned Nogo-A structural gene using the oligodeoxynucleotide 5'-GGT ATC CAT GTT CTT TAA AAG AGG CGC CCT GCG CTA CGG TAG C-3' (the *KasI* recognition site is underlined) (SEQ ID NO: 7), resulting in the vector pASK111-NiFr3. The region encoding the Nogo-A fragment together with the His<sub>6</sub> tag was finally subcloned via *KasI* and *NsiI* (cutting within the vector, downstream of the *Cam<sup>r</sup>* gene) on pASK-IBA4 (Skerra and Schmidt, (2000) *Methods Enzymol.*, 326A, 271-304), which provided the sequence for an N-terminal *Strep*-tag II directly downstream of the OmpA signal sequence. The resulting vector was dubbed pASK111-NiFr4 (SEQ ID NO: 15).

Starting from the human cloned cDNA, the analogous procedure was carried out for cloning of the Nogo-A gene fragments. In doing so, the following gene fragments comprised in the vector pASK75strepII (which differ from the vector pASK75 described in DE 44 17 598 A1 only by use of a sequence coding for the StrepTag® II (Schmid et al, supra) instead of the StrepTag ) were obtained: (1.) A fragment encoding the amino acids 246 to 966 of the full length Nogo-A fused at its N-terminus to the OmpA signal peptide with the introduction of an additional aspartate codon in between (i.e. in front of residue 246) and fused at its C-terminus to the *Strep*-tag II. (2.) A fragment encoding amino acids 334 to 966 of the full length Nogo-A fused at its N-terminus to the OmpA signal peptide with the introduction of an additional glutamine codon in between (i.e. in front of residue 246) and fused at its C-terminus to the *Strep*-tag II

#### Example 2: Bacterial production of soluble Nogo-A fragments (a soluble Nogo-A domain)

By use of the vector pASK111 for the production of Nogo-A fragments of the invention, the respective Nogo-A fragment was fused at its N-terminus to the OmpA signal peptide, thus effecting secretion into the bacterial periplasm, where efficient disulphide bond formation is favoured by an oxidizing redox environment. As explained in Example 1, in case of the rat protein, the bacterial signal peptide was precisely fused to the N-terminus, i.e. residue 174 and 233, respectively, whereas an intermediate amino acid was present between the N-terminal amino acid of the human truncated protein (residue 246 and 334, respectively) and the C-terminus of the signal peptide. At the C-terminus (i.e. following residue 940 of the rat protein, and residue 966 of the human protein) the fragment was

fused with the *Strep*-tag affinity peptide, conferring binding activity towards streptavidin for simplified purification. Transcription of the resulting hybrid gene was under tight control of the tetracycline promoter/operator.

- 5 Cultures of *E. coli* JM83 transformed with the respective expression vector pASK111 obtained in Example 1 were grown in 2 l Luria-Bertani (LB) medium supplemented with chloramphenicol as antibiotic at 22 °C and 200 rpm. Gene expression was induced at an optical density of 0.5 at 550 nm by addition of 400 µg/L anhydrotetracycline (aTc; Acros Organics, Geel, Belgium). After 3 h induction the bacteria were harvested by
- 10 centrifugation and the periplasmic protein fraction was prepared as described by Skerra and Schmidt, supra, with the exception that 200 µg/ml lysozyme were also added to the cell fractionation buffer (50 mM NaPi, pH 7.5, 500 mM sucrose, 1 mM EDTA) for improved release of the Nogo-A fragments.
- 15 All Nogo-A fragments (NI-Fr1 (SEQ ID NO: 16), NI-Fr2 (SEQ ID NO: 17)) of the rat protein as well as corresponding human polypeptides) were purified from the periplasmic protein extract via the *Strep*-tag fused to their C-termini employing streptavidin affinity chromatography (Skerra and Schmidt, supra), whereby elution was effected under mild conditions in the presence of desthiobiotin. After dialysis against chromatography buffer
- 20 (50 mM NaPi, pH 7.5, 150 mM NaCl, 1 mM EDTA) and concentration (Vivaspin 15, MWCO 30 kDa; Greiner, Frickenhausen, Germany) of the eluate further purification was achieved by gel filtration on a Superdex 200 prep grade column (Pharmacia, Uppsala, Sweden) using Dynamax SD-300 HPLC equipment (Rainin, Woburn, MA). NI-Fr4 (SEQ ID NO: 18) was first purified by means of the His<sub>6</sub> tag via IMAC (Skerra, *Gene*, 141,
- 25 (1994a) 79-84) using 50 mM NaPi, pH 7.5, 1 M NaCl as chromatography buffer and a linear elution gradient from 0 to 75 mM imidazole•HCl. The specifically eluted protein fraction was then subjected to streptavidin affinity chromatography as above.

- The yields of purified recombinant rat Nogo-proteins from 2 L shaker-flask experiments
- 30 were highly reproducible and varied between 0.1 and 0.3 mg L<sup>-1</sup> OD<sup>-1</sup> for the Nogo-A fragments. After purification the proteins were stored in PBS (4 mM KH<sub>2</sub>PO<sub>4</sub>, 16 mM Na<sub>2</sub>HPO<sub>4</sub>, 115 mM NaCl) containing 0.1 mM EDTA at 4 °C for up to several weeks. Protein purity was checked by SDS-PAGE using 0.1 % (w/v) SDS, 10 % or 15 % (w/v) polyacrylamide gels (Fling and Gregerson, (1986) *Anal. Biochem.*, 155, 83-88) stained
- 35 with Coomassie brilliant blue. The concentration of the purified recombinant proteins of rat origin was determined using calculated absorption coefficients at 280 nm (Gill and von

Hippel, (1989) *Anal. Biochem.*, **182**, 319-326) of 0.41 ml mg<sup>-1</sup> cm<sup>-1</sup> for the Nogo-A fragments.

5 As shown in Fig.1, the polypeptide comprising residues 174 to 940 (containing 767 residues, i.e. 66 % of full length Nogo-A) was first used for production as a recombinant protein.

10 Upon induction of gene expression NI-Fr1 (SEQ ID NO: 16) was readily liberated from the periplasmic protein fraction of *E. coli* and purified by streptavidin affinity chromatography in one step. SDS PAGE analysis revealed that ca. 50 % of the recombinant protein comprised a product with the proper length whereas 50 % corresponded to a series of smaller polypeptides, probably representing proteolytic degradation products (not shown). In particular, there appeared one prominent band just underneath that for the major recombinant protein. Both bands were subjected to N-terminal sequencing. The upper band 15 yielded the sequence Glu-Thr-Leu-Phe-Ala, which resulted from the precise cleavage of the OmpA signal peptide. The lower band started with the amino acids Ser-Phe-Lys-Glu-His, i.e. at a position 59 codons downstream within the cloned sequence (beginning at residue 233 in the full length primary structure). Its appearance was most likely due to the action of a bacterial protease and might indicate that the N-terminal part of the chosen 20 Nogo-A fragment still belongs to a polypeptide segment devoid of well-defined structure.

In order to achieve better homogeneity of the gene product the first 59 residues of the mature polypeptide chain were deleted from the cloned coding region, leading to NI-Fr2 (SEQ ID NO: 17) (cf. Fig.1A). This protein was readily produced in the periplasm of *E.* 25 *coli*, with similar yields as the former version but clearly reduced degradation pattern. The possible presence of structural disulphide bonds in the recombinant protein was investigated by individually substituting all eight Cys residues (corresponding to positions 323, 403, 443, 536, 574, 676, 885, and 890 in the full length Nogo-A sequence) with Ser via site-directed mutagenesis. The eight mutant Nogo-A fragments were produced in *E.* 30 *coli* as before. However, it was not possible to recover the mutants Cys<sup>323</sup>ØSer and Cys<sup>885</sup>ØSer from the periplasmic protein fraction, while the mutants Cys<sup>443</sup>ØSer and Cys<sup>890</sup>ØSer gave rise to significantly diminished yields after *Strep*-tag purification when compared with the wild-type protein. In contrast, the other four mutants were produced at similar amounts as the original Nogo-A fragment. These observations indicate that at least 35 some of the Cys residues are important for folding and may be involved in cystine crosslinks.

The wild-type NI-Fr2 (SEQ ID NO: 17) protein still gave rise to certain truncated products, which was considered undesirable for precise binding measurements (see below). Therefore, a doubly tagged version of the recombinant protein was prepared using an otherwise identical expression system. First, the *Strep*-tag at the C-terminus was  
5 exchanged by a His<sub>6</sub>-tag (yielding NI-Fr3 as an intermediate construct, not shown), and, second, the *Strep*-tag was inserted at the N-terminus again, downstream of the OmpA signal peptide. Interestingly, the yield of bacterially produced soluble protein, termed NI-Fr4 (SEQ ID NO: 18) (cf. Fig.1A), was found to be significantly higher (by a factor of 2.5, approaching 300  $\mu\text{g L}^{-1}$  OD<sup>-1</sup>). NI-Fr4 (SEQ ID NO: 18) was isolated from the  
10 periplasmic protein fraction in two steps by immobilized metal affinity chromatography (IMAC) followed by streptavidin affinity chromatography as described above. This protein was essentially pure, just a minor fraction of truncated polypeptide chains was still detectable (Fig.1B).

15 Furthermore, a mutant of NI-Fr2 devoid of Cys<sup>574</sup> and Cys<sup>676</sup> was also produced as described above and used as Nogo protein in the affinity maturation of antibody fragments directed to Nogo-A (Example 5).

20 Thus, the invention provides for the first time soluble and stable Nogo-A polypeptides which can be used for the detailed elucidation of the biological role of the Nogo-A protein and in the identification of substances with binding affinity to Nogo-A. This identification method will be demonstrated in the following Examples.

25 Example 3: Identification of antibody fragments derived from IN-1 with improved binding affinity to Nogo-A

The IN-1 Fab fragment with variable domains derived from the mouse monoclonal antibody IN-1 (Bandtlow et al., 1996, supra) and human constant domains belonging to the subclass IgG1/ $\kappa$  (Schiweck and Skerra, (1995) *Proteins: Struct. Funct. Genet.*, 23, 561-  
30 565) was used as starting molecule for the identification of antibody fragments with improved affinity and neutralizing effect on the neurite-growth-inhibiting activity of Nogo-A. The IN-1 muteins used in the method of identifying new binding molecules were either derived from a computer-based modeling study or an evolutionary approach. The  
35 following general methodology was used for construction of the respective genes and the production antibody fragments.

Vector construction for Fab fragments

The IN-1 Fab fragment and its mutants were produced utilizing the vectors pASK88, pASK106 or pASK107. All of them encode a chimeric Fab fragment with variable domains derived from the mouse monoclonal antibody IN-1 and human constant domains belonging to the subclass IgG1/ $\kappa$  (see above). Secretion into the oxidizing milieu of the bacterial periplasm is ensured by the presence of signal peptides at the N-termini of both chains (Skerra, 1994a, supra) and transcription of the artificial dicistronic operon is under tight control of the chemically inducible *tetP*<sup>o</sup> (Skerra, *Gene*, (1994b) 151, 131-135). pASK88 (Schiweck and Skerra, supra) was used for soluble expression and purification via the His<sub>6</sub> tag attached to the C-terminus of the heavy chain (Fiedler and Skerra, (2001a) In Kontermann, R. and Dübel, S. (eds.), *Antibody Engineering*. Springer Verlag, Heidelberg, pp. 243-256; Skerra, 1994b), whereas pASK107 provided the *Strep*-tag II for streptavidin affinity purification instead. pASK106 codes for a Fab fragment similarly as pASK88 but with an albumin-binding domain (ABD) appended to the C-terminus of the light chain (König and Skerra, (1998) *J. Immunol. Methods*, 218, 73-83). The variable domain genes were exchanged between the differing vector formats using conserved restriction sites as described (Skerra, 1994a).

Single amino acid exchanges within the IN-1 Fab fragment or its mutants were introduced by site-directed mutagenesis. For this purpose single-stranded DNA of the corresponding vectors pASK88-IN1 or pASK88-I.2.6 (see below) was used in conjunction with appropriate oligodeoxynucleotide primers.

Random amino acid substitutions used for the generation of the genetic random library of Example 3.2 were introduced into the variable domain (V<sub>L</sub>) gene of the IN-1 light chain at defined positions via PCR by means of degenerate oligodeoxynucleotide primers (without the phosphorothioate modification) in conjunction with *Taq* DNA polymerase. Amplification was performed on pASK85-IN1 with the originally cloned genes (Bandtlow et al., 1996, supra) as template. The forward primer 5'-GAC ATT GAG CTC ACC CAG TCT CCA GCA ATC ATG KCT GC-3' (SEQ ID NO. 8) (*Sst*I restriction site underlined) was used in all experiments whereas the oligodeoxynucleotide 5'-GCG CTT CAG CTC GAG CTT GGT CCC AGC TCC GAA CGT MNN AGG MNN MNN TAA CACATT TTG ACA GTA-3' (SEQ ID. NO. 9) (*Xho*I restriction site underlined) served as backward primer for randomizing the CDR-L3 positions L93, L94, and L96 at the first stage of the affinity maturation process (see below, Example 3.2). The second mutagenesis cycle was performed with pASK88-I.2.6(L96V) as template and the oligodeoxynucleotide 5'-GCG CTT CAG CTC GAG CTT GGT CCC AGC TCC GAA CGT AAC CGG CAC CCG



MNN MNN ATT TTG ACA GTA ATA CGT TGC-3' (SEQ ID NO: 10) as second primer for randomizing the positions L91 and L92 together with fixed mutations at L93, L94, and L96. In each case a single PCR product was obtained, purified from a 1 % agarose gel, and cut with *Sst*I and *Xho*I. The resulting DNA fragment of approximately 300 bp was ligated with the likewise cut vector backbone of pASK106-IN1 (cf. above). Colonies obtained after transformation of CaCl<sub>2</sub>-competent *E. coli* K-12 JM83 cells (Yanisch-Perron et al., (1985) *Gene*, 33, 103-119) were directly subjected to the filter-sandwich colony screening assay.

#### 10 Bacterial production of Fab fragments

Cultures of *E. coli* JM83 transformed with the respective derivatives of vectors pASK88, pASK106, and pASK 107 were grown in 2 l Luria-Bertani (LB) medium supplemented with ampicillin at 22 °C and 200 rpm. Gene expression was induced at an optical density of 0.5 at 550 nm by addition of 200 µg/L anhydrotetracycline (aTc; Acros Organics, Geel, Belgium). After 3 h induction the bacteria were harvested by centrifugation and the periplasmic protein fraction was prepared as described by Skerra and Schmidt, supra.

The recombinant IN-1 Fab fragments were purified either by IMAC via the His<sub>6</sub> tag fused to the C-terminus of their heavy chain (Fiedler and Skerra, 2001a, supra) or, when using pASK107 (cf. above), via streptavidin affinity chromatography (Schlapschy and Skerra, (2001) In Kontermann, R. and Dübél, S. (eds.) Antibody Engineering. Springer Verlag, Heidelberg, pp. 292-306). IMAC was also performed under FPLC conditions using a POROS MC/M column (0.46 cm x 10 cm; PerSeptive Biosystems, Wiesbaden, Germany) charged with Zn<sup>2+</sup> ions and Dynamax SD-300 HPLC equipment (Rainin, Woburn, MA) operating at a flow rate of 2.0 ml/min. 12.5 ml of periplasmic extract from a 2 L *E. coli* culture dialyzed against 50 mM NaP<sub>i</sub>, pH 7.5, 500 mM betaine was applied to the column and, after washing with dialysis buffer, elution was effected by application of a linear gradient of 200 mM imidazole•HCl, pH 7.5, 50 mM NaP<sub>i</sub>, 500 mM betaine against dialysis buffer. This method enabled a five-fold quicker purification compared with the conventional procedure of Fiedler and Skerra, 2001a, supra, yielding recombinant Fab fragments with an apparent purity of >95 % as estimated from SDS-PAGE. The yields of purified recombinant proteins from 2 L shaker-flask experiments were highly reproducible and varied between 0.04 and 0.8 mg L<sup>-1</sup> OD<sup>-1</sup> for the different Fab fragments.

Example 3.1: Identification of antibody fragments with improved binding affinity to Nogo-A based on computer modeling

Experiments on the detection of natural Nogo-A on Western blots or on tissue sections by means of the bacterially produced IN-1 F<sub>ab</sub> fragment revealed relatively weak signals (Bandtlow et al., 1996, supra), indicating that the antigen affinity was poor.

A computer-modeling study was first carried out order to select candidate molecules to be tested in the identification method of the present invention. This modeling study was based on a human anti-thyroid peroxidase autoantibody (Protein Data Bank (PDB) entry 1VGE) and a murine anti-phenylarsonate antibody (PDB entry 6FAB), both of which have sets of CDRs with the same lengths and canonical structure determinants as IN-1 and share a high amino acid sequence similarity with it. This analysis revealed that the CDR-L3 of IN-1 and, to a lesser extent, its CDR-L1 appeared to be the most promising target regions for protein engineering towards improved antigen recognition. Especially residue L96 and also residue L32 (in CDR-L1) appeared to be exposed close to the center of the combining site and thus to be possibly involved in contacts with the antigen.

Within CDR-L1 both IN-1 and 1VGE have an Ala residue at position L32 whereas 6FAB carries a Phe. On the other hand, IN-1 as well as 6FAB carry an Arg at position L96 (in CDR-L3) while 1VGE exhibits a Leu. Therefore, the structural consequences of the amino acid exchanges A<sup>L32</sup>ØF and R<sup>L96</sup>ØL within the V<sub>L</sub> domain of IN-1 were modeled, resulting in their identification as potential paratope residues. The corresponding single amino acid exchanges in the recombinant F<sub>ab</sub> fragment were introduced by site-directed mutagenesis followed by production in *E. coli* and purification via IMAC as described above. A test for neutralizing biological activity in the 3T3 fibroblast assay for inhibition of cell spreading on a CNS myelin substrate (Bandtlow et al., 1996, supra) revealed that the mutant R<sup>L96</sup>ØL had a slightly improved activity. In contrast, the mutant A<sup>L32</sup>ØF had mostly lost its neutralizing activity when compared with the wild-type IN-1 F<sub>ab</sub> fragment (data not shown).

Example 3.2: Identification of antibody fragments with improved binding affinity to Nogo-A by *in vitro* affinity maturation of the IN-1 F<sub>ab</sub> fragment

In order to perform functionally more complex changes within the paratope of the IN-1 antibody a cluster of three amino acids in CDR-L3 corresponding to positions L93, L94, and L96 was subjected to targeted random mutagenesis.

All 20 side chains were allowed for substitution in each position, followed by screening for improved binding of the recombinant Nogo-A fragment via a filter-sandwich colony screening assay. This assay was carried out based on published procedures (Skerra et al.,  
5 *Anal. Biochem.*, 196, 151-155; Schlehuber et al., (2000) *J. Mol. Biol.*, 297, 1105-1120).

For this purpose a genetic random library was prepared by PCR amplification of the IN-1 VL gene using the degenerate primer of SEQ ID. NO. 9 that carried the corresponding mixed base positions (see above). The mutagenized gene fragment was recloned on the  
10 expression vector pASK106-IN1 (encoding a Fab fragment fused with an albumin-binding domain to the C-terminus of its light chain; König and Skerra, supra). *E. coli* JM83 was transformed with the ligation mixture and transformed cells harboring the pASK106 vector were plated on a hydrophilic membrane (GVWP, 0.22  $\mu\text{m}$ ; Millipore, Bedford, MA), placed on a petri dish with LB/Amp agar, such that approximately 500 colonies were  
15 obtained, and incubated at 37°C for 8 to 9 h. In the meantime a hydrophobic membrane (Immobilon-P, 0.45  $\mu\text{m}$ ; Millipore) was coated with 10 mg/ml human serum albumin (HSA; Sigma, Deisenhofen, Germany) in PBS for four hours and blocked with 3 % (w/v) BSA (Roth, Karlsruhe, Germany), 0.5 % (v/v) Tween 20 in PBS. The membrane was washed twice with PBS, soaked in LB/Amp containing 200  $\mu\text{g/ml}$  aTc, and placed on an  
20 LB/Amp agar plate supplemented with 200  $\mu\text{g/ml}$  aTc. The first membrane, carrying tiny colonies of the transformed cells, was then placed onto the second (hydrophobic) membrane. The filter sandwich was incubated for 16 h at 22 °C. During this period the mutated IN-1 Fab fragments became secreted – and partially released from the colonies by leakage from the bacterial periplasm – and finally immobilized on the lower membrane via  
25 complex formation between HSA and ABD.

The first membrane with the still viable colonies was transferred to a fresh LB/Amp agar plate and stored at 4 °C. The second membrane was washed three times in PBS containing 0.1 % (v/v) Tween 20 (PBS/T) and the immobilized Fab fragments, each in a spot  
30 corresponding to the position of the original colony, were probed for antigen binding. To this end recombinant Nogo-A fragment NI-FR2 was labeled at a molar ratio of 5:1 with digoxigenin-3-O-methylcarbonyl-e-aminocaproic acid N-hydroxy-succinimide ester (Roche Diagnostics, Mannheim, Germany) and applied to the membrane for one hour at a concentration of 30 or 50  $\mu\text{g/ml}$  in PBS/T. After washing three times with PBS/T the  
35 membrane was incubated for one hour with 0.75 u/ml anti-digoxigenin Fab fragment conjugated with alkaline phosphatase (Roche Diagnostics) in 10 ml PBS/T. The membrane was finally washed twice with PBS/T and twice with PBS and the signals were developed

using standard chromogenic substrates as described (Schlehuber et al., supra). Colonies corresponding to signals with an intensity above average were identified, recovered from the first membrane, and propagated for further analysis of their recombinant gene product.

- 5 In total, the cell suspension containing transformed *E. coli* JM83 cells harboring the pASK106 vector was plated on four filter membranes, placed on top of agar plates, thus screening approximately 2000 colonies in parallel. From colonies that gave rise to staining signals above average 31 clones were recovered, propagated, and their plasmids were isolated for DNA sequence analysis. Out of these 31 investigated clones, 12 plasmids were  
10 identified carrying functional VL genes (for the mutations see Table 1), whereas otherwise frameshift mutations or internal amber termination codons were abundant.

**Table I:** Mutants obtained from a first affinity maturation based on the IN-1 Fab fragment

	Position					Signal in	Expression	ELISA
	L91	L92	L93	L94	L96	CSA <sup>a</sup>	yield <sup>b</sup>	signal
IN-1 wt	Val	Leu	Ser	Thr	Arg	+	+++	—
I.1.4	— <sup>c</sup>	—	Pro	Val	Trp	+++	+	+
I.1.6	—	—	Asn	Leu	Cys	++		
I.1.11	—	—	Tyr	Thr	Cys	++		
I.1.16	—	—	Met	Cys	Asn	++	+	—
I.2.2	—	—	Arg	Thr	Asn	+++	+++	—
I.2.4	—	—	Gly	Thr	Phe	+++		
I.2.5	—	—	Pro	Cys	Val	+++		
I.2.6	—	—	Arg	Val	Cys	+++	+	+++
I.2.8	—	—	Tyr	Ala	Gly	++	+	—
I.2.9	—	—	Arg	Pro	Pro	++	++	—
I.3.7	—	—	Phe	Arg	Leu	+++	+	—
I.4.4	—	—	Asp	Arg	Leu	+++		
I.2.6 (L96V)	—	—	Arg	Val	Val		+++	+

<sup>a</sup> filter-sandwich colony screening assay; <sup>b</sup> in *E. coli* JM83 using the vector pASK88; <sup>c</sup> no  
15 exchange

#### Example 4: Production of IN-1 muteins

The muteins derived from the variable domains of the antibody IN-1 identified in Example 3.2 were then produced in amounts suitable for characterization of the binding properties of these muteins.

For soluble production of the recombinant F<sub>ab</sub> fragments in a standard format (i.e. without the ABD domain but still having a His<sub>6</sub> tag fused to the C-terminus of the heavy chain) the mutagenized V<sub>L</sub> gene cassettes from seven selected clones (cf. Table I) were subcloned on pASK88-IN1 (Fiedler and Skerra, (1999) *Protein Expr. Purif.*, 17, 421-427). The mutants were produced in shaker flask cultures and isolated from the periplasmic protein fraction in one step via IMAC. All F<sub>ab</sub> fragments contained the light and heavy chains in stoichiometric composition and quantitatively linked via a disulphide bond.

Antigen-binding activity of the mutant F<sub>ab</sub> fragments was subsequently tested by ELISA using the recombinant NI-Fr2 for coating of the microtitre plate wells (Fig.2).

ELISA was carried out in a 96 well microtitre plate (Becton Dickinson, Heidelberg, Germany) at ambient temperature with incubation steps of 1 h unless otherwise stated. Three washing steps with PBS/T were used after each incubation, and residual liquid was removed thoroughly. The wells were coated for 4 h with 50 µl of a solution of NI-Fr2 at concentrations between 180 and 200 µg/ml in PBS buffer and then blocked with 200 µl 3 % (w/v) BSA, 0.5 % (v/v) Tween 20 in PBS. After washing, 50 µl of the purified recombinant F<sub>ab</sub> fragment was applied at a dilution series in PBS/T. The wells were then incubated with 50 µl anti-human C<sub>κ</sub> antibody conjugated with alkaline phosphatase (Sigma), diluted 1:1000 in PBS/T. Signals were finally developed in the presence of p-nitrophenyl phosphate (Voss and Skerra, (1997) *Protein Eng.*, 10, 975-982). Enzymatic activity was measured at 25°C as the change in absorbance at 405 nm per min with a SpectraMAX 250 instrument (Molecular Devices, Sunnyvale, CA). The data were corrected for background values determined in wells that were merely coated with BSA and fitted by non-linear least squares regression as described by Voss and Skerra, *supra*.

Almost no binding signal above background was obtained with the recombinant wild-type IN-1 F<sub>ab</sub> fragment, illustrating its low antigen affinity. In contrast, the mutant I.2.6 (cf. Fig.2A) gave rise to a clearly detectable and concentration-dependent binding signal. No significant signal was obtained in a control experiment with BSA serving as antigen.

Hence, the mutant I.2.6 was the protein of choice for further affinity maturation experiments.

Example 5: Affinity maturation of the mutant I.2.6

Unfortunately, the I.2.6 mutant of the IN-1 Fab fragment was produced as a soluble protein in *E. coli* at a much lower level, with a relative yield of 5 % after purification (0.04 mg L<sup>-1</sup> OD<sup>-1</sup> vs. 0.8 mg L<sup>-1</sup> OD<sup>-1</sup> for the wild-type IN-1 Fab fragment). Obviously, the free Cys residue that occurred at the exposed position L96 within CDR-L3 had a deleterious influence on the folding efficiency of the Ig fragment and a concomitant toxic effect on the bacterial host cell, as it had been similarly observed in other cases. Following earlier substitution experiments concerning position L96 (cf. above) attempts were made to replace the Cys residue in the I.2.6 mutant by small apolar side chains such as those of Ala, Val, Met, Leu, and Ile. The substitutions were introduced by site-directed mutagenesis and all corresponding recombinant Fab fragments were produced and purified as before, resulting in yields that were similar again to the wild type IN-1 Fab fragment. However, when binding activity towards the recombinant NI-Fr2 antigen was tested in an ELISA as described above, all these mutants gave rise to significantly lower signals than the original I.2.6 Fab fragment. Merely the replacement Cys<sup>L96</sup>→Val (cf. Fig.2) resulted in a detectable binding behavior and was therefore used as basis for the second affinity maturation cycle.

CDR-L3 forms a connecting loop between two neighboring beta-strands such that the positions L91 and L92 are in close spatial proximity with L96. Hence, in order to structurally compensate a possible misfit at position L96 – due to the exchange of Cys by Val – the positions L91 and L92 within CDR-L3 of the I.2.6(L<sup>96</sup>Val) Fab fragment were subjected to targeted random mutagenesis using the oligonucleotide of SRQ ID NO: 9 and the filter-sandwich colony screening assay was performed again. This time the stringency of selection was raised by lowering the concentration of the recombinant antigen – a mutant of NI-Fr2 devoid of Cys<sup>574</sup> and Cys<sup>676</sup> – from 50 µg/ml to 30 µg/ml. From screening approximately 1000 colonies spread on two filter membranes, 16 clones were identified according to their pronounced color signals. In contrast with the previous experiment all of them carried plasmids encoding functional mutants of the I.2.6(L<sup>96</sup>V) Fab fragment. The VL gene cassettes of four clones (cf. Table 2) were subcloned on pASK88-IN1 and the corresponding Fab fragments were produced and purified as before. One of them, the II.1.8 Fab fragment (cf. Fig.2A), exhibited clearly improved binding activity over the I.2.6(L<sup>96</sup>V) mutant in an ELISA (Fig.2B), even though its affinity was still lower than that

of the original I.2.6 mutant carrying the free Cys residue. Nevertheless, the yield of the II.1.8 mutant was 12-fold higher upon expression in *E. coli* and thus close to that of the recombinant wild-type IN-1 Fab fragment (0.5 vs. 0.8 mg L<sup>-1</sup> OD<sup>-1</sup>, respectively).

- 5 Table 2: Mutants obtained from a second affinity maturation based on the I.2.6-Fab fragment

	Position					Signal in	Expression	ELISA
	L91	L92	L93	L94	L96	CSA <sup>a</sup>	yield <sup>b</sup>	signal
IN-1 wt	Val	Leu	Ser	Thr	Arg	+	+++	-
I.2.6 (L96V)	-	-	Arg	Val	Val		+++	+
II.1.1	Arg	Lys	Arg	Val	Val	+++	+++	-
II.1.3	Met	Lys	Arg	Val	Val	++	+++	-
II.1.7	Leu	Lys	Arg	Val	Val	++	+++	-
II.1.8	Ile	Asn	Arg	Val	Val	++	+++	++

<sup>a</sup> filter-sandwich colony screening assay; <sup>b</sup> in *E. coli* JM83 using the vector pASK88; <sup>c</sup> no exchange

#### 10 Example 6: Functional analysis of engineered Fab fragments

For a detailed analysis of the antigen-binding activity and application in immunohistochemistry as well as cell culture assays the different engineered versions of the IN-1 Fab fragment were produced in *E. coli* in shaker flask cultures and purified by IMAC to homogeneity (Fig.3a).

15 The thermodynamic affinity for the recombinant Nogo-A fragment NI-Fr4 was determined both for the II.1.8 mutant and for the wild-type IN-1 Fab fragment using the method of real time surface plasmon resonance (SPR) on a Biacore-X<sup>®</sup> system equipped with an Ni/NTA-derivatized sensor chip<sup>®</sup> (Biacore AB, Uppsala, Sweden). PBS containing 0.005 % (v/v) surfactant P20 was used as continuous flow buffer as well as for dilution of proteins. Analysis was performed at 25 °C using a flow rate of 35 µl/min.

25 For each measurement the derivatized chip surface was charged with 70 µl 0.5 mM NiSO<sub>4</sub>, followed by immobilization of NI-Fr4 via its His<sub>6</sub> tag in one of the two flow channels by applying 70 µl of a 50 µg/ml solution of the purified recombinant protein.

Then the F<sub>ab</sub> fragment (produced by means of the vector pASK107 and purified via the *Strep*-tag II; see Example 1)) was injected at a defined concentration (between 0.25 and 6.8 μM) for 2 minutes, followed by buffer flow for 4 minutes. The chip surface was regenerated using 70 μl 0.35 M EDTA, pH 8.0 in flow buffer prior to the next measurement. Each time-dependent binding isotherm of the F<sub>ab</sub> fragment was corrected for the background signal that was detected in the flow channel without NI-Fr4 using BIAevaluation software (Version 3.0). Resonance unit values for the bound F<sub>ab</sub> fragment at equilibrium for each applied concentration were then deduced and fitted (Voss and Skerra, supra) by non-linear least squares regression using an equation of the type  $y=a*x/(b+x)$ .

By this way binding isotherms were obtained for the wild-type and engineered F<sub>ab</sub> fragments (Fig.3B), from which dissociation constants were deduced. The K<sub>D</sub> value for the recombinant wild-type IN-1 F<sub>ab</sub> fragment was  $7.8 \pm 1.9$  μM. In contrast, the dissociation constant for its II.1.8 mutant was  $1.04 \pm 0.18$  μM, i.e. 8-fold better. Control experiments with an unrelated protein, recombinant cystatin carrying a His<sub>6</sub>-tag, that was used instead of the Nogo-A fragment for coating of the sensor chip confirmed absence of unspecific binding (not shown).

#### Example 7: Use of engineered IN 1-F<sub>ab</sub> fragments for detection of natural Nogo-A

The engineered II.1.8 F<sub>ab</sub> fragment was further employed for the detection of natural Nogo-A by immunohistochemistry.

For this purpose, cryosections (12 μm) of rat brain (*Rattus norvegicus*) were fixed for 10 minutes using ice-cold ethanol. The following incubation steps were then each performed for 1 h at room temperature in a humid chamber using PBS. Unless otherwise stated slides were washed for 5 min with PBS. After blocking with 4 % (w/v) BSA the F<sub>ab</sub> fragment (produced using the pASK88 vector type and purified via the His<sub>6</sub> tag) was applied at a concentration of 100 μg/ml. After three washing steps bound F<sub>ab</sub> fragment was detected with an anti-human C<sub>k</sub> antibody alkaline phosphatase conjugate (Sigma), diluted 1:100. The sections were then washed three times with TBS (25 mM Tris/HCl, pH 7.4, 145 mM NaCl, 3 mM KCl) and staining was performed using a "Fast Red" kit (Roche Diagnostics). The microscopic slides were photographed on an Axiophot microscope (Carl Zeiss, Jena, Germany) using 10- or 20-fold magnification.

Fig.4 shows cross sections of adult rat brain which were stained with different recombinant F<sub>ab</sub> fragments, followed by the above-mentioned secondary antibody conjugated with a



reporter enzyme. The II.1.8 mutant specifically stained the myelinated regions, especially the *Corpus callosum* and transected fiber bundles of the *Capsula interna* in the *Corpus striatum*. The staining pattern is similar in morphology and intensity to the one obtained with a recombinant F<sub>ab</sub> fragment derived from the monoclonal antibody 8-18C5, which is directed against the major oligodendrocyte glycoprotein MOG (Linington et al., (1984) *J. Neuroimmunol.*, 6, 387-396). The staining with the recombinant wild-type IN-1 F<sub>ab</sub> fragment was very weak under the present conditions of fixation. An unrelated recombinant anti-CD30 F<sub>ab</sub> fragment derived from the HRS-3 antibody (Engert et al., (1990) *Cancer Res.*, 50, 2929-2935) gave only background staining. These results demonstrate that the affinity of the II.1.8 mutant of the IN-1 F<sub>ab</sub> fragment has been raised by use of truncated Nogo-proteins of the invention to a sufficient extent in order to detect the Nogo-A antigen in standard immunochemical experiments. Analogous data were obtained using immunofluorescence microscopy (not shown).

Example 8: Neutralization of the neurite-growth-inhibiting activity of Nogo-A by engineered IN 1-F<sub>ab</sub> fragments

Finally, the engineered F<sub>ab</sub> fragments were tested for their neutralizing effect on Nogo-A substrate properties using a cell culture assay.

Neurite growth-modulating properties of the different F<sub>ab</sub> fragments were tested on 4-well plastic dishes (Greiner, Nürtingen, Germany) coated with recombinant Nogo-A. The test wells were coated for 20 min with 100 µg/ml poly-L-lysine, washed with Hank's balanced salt solution (HBSS; Life Technologies, Basel, Switzerland) and coated for 2 h with 15 or 30 µg/ml of recombinant rat Nogo-A (Chen et al., supra). Recombinant Nogo-A was omitted in the wells serving for control. After aspiration, the wells were washed with Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10 % v/v fetal calf serum (FCS; Life Technologies) and blocked in the same medium for 20 min at 37 °C.

Cerebellar cell cultures were prepared from rat cerebella on postnatal day 7/8. Cells were dissociated by combined trituration and trypsinization and purified on Percoll gradients as described (Hatten, *J. Cell Biol.*, 100, 384-396). The cerebellar granule cells were plated in chemically defined neurobasal medium supplemented with B27 and 0.2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Life Technologies). To assess the neutralization of inhibitory activity, substrate-coated wells were first incubated with 100 µg/ml of the different recombinant F<sub>ab</sub> fragments dialyzed against NaCl/P<sub>i</sub> (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) for 20 min at 37 °C. The

wells were then washed briefly with HBSS and cells were applied in the presence of the F<sub>ab</sub> fragments.

Assays were stopped after 24 h in culture by adding 4 % (w/v) formalin buffered with NaCl/P<sub>i</sub>. For assaying the inhibitory substrate properties, the proportion of total cells bearing neurites longer than the diameter of the cell body (indicating that neurite outgrowth was successfully initiated) was determined. Under control conditions, i.e. in the absence of recombinant Nogo-A, 70 % of the cerebellar granule neurons formed processes. Quantification of neurite lengths was performed on cultures monitored with a Zeiss Axiophot microscope. Phase contrast pictures were acquired with a 12-bit digital CCD camera (Visicam Visitron, Germany) and analyzed using Metamorph software (Universal Imaging Corporation, West Chester, PA). For each well the longest neurites of at least 100 isolated neurons were measured and averaged. Three wells were investigated for each experimental condition.

As shown in Fig.5, neurite outgrowth of cerebellar granule cells was severely reduced when recombinant Nogo-A was used as a substrate. In contrast, poly-L-lysine promoted extensive attachment of granule cells in its absence as well as robust neurite growth with an average neurite length of approximately 70 µm in 70 % of adherent cells. In this *in vitro* bioassay functional neutralisation of the inhibitory Nogo-A substrate was observed at different degrees for the various engineered F<sub>ab</sub> fragments (Fig.5). While the recombinant wild-type IN-1 F<sub>ab</sub> fragment revealed partial neutralization of Nogo-A activity, as previously demonstrated (Bandtlow et al., 1996 supra), introduction of the mutation Ala<sup>L32</sup>ØPhe into the V<sub>L</sub> domain completely abolished this effect. In contrast, the mutants I.2.6(L<sup>96</sup>V) and, in particular, II.1.8 exhibited significantly stronger neutralizing effects, as revealed by their better fibre growth-promoting activities, even when the concentration of the inhibitory material was raised. None of the applied F<sub>ab</sub> fragments exerted an effect on neurite outgrowth of cerebellar granule cells under control conditions, i.e. in the absence of Nogo-A. Notably, the stepwise improvement of the biological activity of the mutants I.2.6(L<sup>96</sup>V) and II.1.8 in comparison with the wild-type IN-1 F<sub>ab</sub> fragment correlated well with their relative increase in antigen affinity observed in the ELISA experiment (Fig.2B).

Accordingly, the soluble truncated Nogo-A fragments according to the present invention provide for an assay system which allows identification of substances which neutralize the inhibitory activity of Nogo-A and which thus can be used as diagnostic and therapeutic agent.

## Sequence listing

<110> Pieris Proteolab AG

<120> Soluble truncated polypeptides of the Nogo-A protein,  
methods for the production of such polypeptides and methods for  
identifying compounds having detectable affinity to a Nogo-A  
protein

<160> 18

<210> 1

<211> 1163

<212> PRT

<213> rat

<220>

<223> rat Nogo-A protein

<400> 1

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			20						25					30

Glu	Pro	Glu	Asp	Glu	Glu	Asp	Glu	Glu	Glu	Glu	Glu	Asp	Glu	Glu
			35						40					45

Glu	Asp	Asp	Glu	Asp	Leu	Glu	Glu	Leu	Glu	Val	Leu	Glu	Arg	Lys
			50						55					60

Pro	Ala	Ala	Gly	Leu	Ser	Ala	Ala	Ala	Val	Pro	Pro	Ala	Ala	Ala
			65						70					75

Ala	Pro	Leu	Leu	Asp	Phe	Ser	Ser	Asp	Ser	Val	Pro	Pro	Ala	Pro
			80						85					90

Arg	Gly	Pro	Leu	Pro	Ala	Ala	Pro	Pro	Ala	Ala	Pro	Glu	Arg	Gln
			95						100					105

Pro	Ser	Trp	Glu	Arg	Ser	Pro	Ala	Ala	Pro	Ala	Pro	Ser	Leu	Pro
			110						115					120

Pro	Ala	Ala	Ala	Val	Leu	Pro	Ser	Lys	Leu	Pro	Glu	Asp	Asp	Glu
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Pro	Pro	Ala	Arg	Pro	Pro	Pro	Pro	Pro	Pro	Ala	Gly	Ala	Ser	Pro
			140						145					150

Leu	Ala	Glu	Pro	Ala	Ala	Pro	Pro	Ser	Thr	Pro	Ala	Ala	Pro	Lys
			155						160					165

Arg	Arg	Gly	Ser	Gly	Ser	Val	Asp	Glu	Thr	Leu	Phe	Ala	Leu	Pro
			170						175					180

Ala	Ala	Ser	Glu	Pro	Val	Ile	Pro	Ser	Ser	Ala	Glu	Lys	Ile	Met
			185						190					195

Asp	Leu	Met	Glu	Gln	Pro	Gly	Asn	Thr	Val	Ser	Ser	Gly	Gln	Glu
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

200					205					210				
Asp	Phe	Pro	Ser	Val	Leu	Leu	Glu	Thr	Ala	Ala	Ser	Leu	Pro	Ser
				215										225
Leu	Ser	Pro	Leu	Ser	Thr	Val	Ser	Phe	Lys	Glu	His	Gly	Tyr	Leu
				230										240
Gly	Asn	Leu	Ser	Ala	Val	Ser	Ser	Ser	Glu	Gly	Thr	Ile	Glu	Glu
				245										255
Thr	Leu	Asn	Glu	Ala	Ser	Lys	Glu	Leu	Pro	Glu	Arg	Ala	Thr	Asn
				260										270
Pro	Phe	Val	Asn	Arg	Asp	Leu	Ala	Glu	Phe	Ser	Glu	Leu	Glu	Tyr
				275										285
Ser	Glu	Met	Gly	Ser	Ser	Phe	Lys	Gly	Ser	Pro	Lys	Gly	Glu	Ser
				290										310
Ala	Ile	Leu	Val	Glu	Asn	Thr	Lys	Glu	Glu	Val	Ile	Val	Arg	Ser
				305										315
Lys	Asp	Lys	Glu	Asp	Leu	Val	Cys	Ser	Ala	Ala	Leu	His	Ser	Pro
				320										330
Gln	Glu	Ser	Pro	Val	Gly	Lys	Glu	Asp	Arg	Val	Val	Ser	Pro	Glu
				335										345
Lys	Thr	Met	Asp	Ile	Phe	Asn	Glu	Met	Gln	Met	Ser	Val	Val	Ala
				350										360
Pro	Val	Arg	Glu	Glu	Tyr	Ala	Asp	Phe	Lys	Pro	Phe	Glu	Gln	Ala
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Trp	Glu	Val	Lys	Asp	Thr	Tyr	Glu	Gly	Ser	Arg	Asp	Val	Leu	Ala
				380										390
Ala	Arg	Ala	Asn	Val	Glu	Ser	Lys	Val	Asp	Arg	Lys	Cys	Leu	Glu
				395										405
Asp	Ser	Leu	Glu	Gln	Lys	Ser	Leu	Gly	Lys	Asp	Ser	Glu	Gly	Arg
				410										420
Asn	Glu	Asp	Ala	Ser	Phe	Pro	Ser	Thr	Pro	Glu	Pro	Val	Lys	Asp
				425										435
Ser	Ser	Arg	Ala	Tyr	Ile	Thr	Cys	Ala	Ser	Phe	Thr	Ser	Ala	Thr
				440										450
Glu	Ser	Thr	Thr	Ala	Asn	Thr	Phe	Pro	Leu	Leu	Glu	Asp	His	Thr
				455										465
Ser	Glu	Asn	Lys	Thr	Asp	Glu	Lys	Lys	Ile	Glu	Glu	Arg	Lys	Ala
				470										480
Gln	Ile	Ile	Thr	Glu	Lys	Thr	Ser	Pro	Lys	Thr	Ser	Asn	Pro	Phe
				485										495
Leu	Val	Ala	Val	Gln	Asp	Ser	Glu	Ala	Asp	Tyr	Val	Thr	Thr	Asp
				500										510
Thr	Leu	Ser	Lys	Val	Thr	Glu	Ala	Ala	Val	Ser	Asn	Met	Pro	Glu

	515		520		525
Gly Leu Thr Pro	Asp Leu Val Gln Glu	Ala Cys Glu Ser Glu	Leu		
	530		535		540
Asn Glu Ala Thr	Gly Thr Lys Ile Ala	Tyr Glu Thr Lys Val	Asp		
	545		550		555
Leu Val Gln Thr	Ser Glu Ala Ile Gln	Glu Ser Leu Tyr Pro	Thr		
	560		565		570
Ala Gln Leu Cys	Pro Ser Phe Glu Glu	Ala Glu Ala Thr Pro	Ser		
	575		580		585
Pro Val Leu Pro	Asp Ile Val Met Glu	Ala Pro Leu Asn Ser	Leu		
	590		595		600
Leu Pro Ser Ala	Gly Ala Ser Val Val	Gln Pro Ser Val Ser	Pro		
	605		610		615
Leu Glu Ala Pro	Pro Pro Val Ser Tyr	Asp Ser Ile Lys Leu	Glu		
	620		625		630
Pro Glu Asn Pro	Pro Pro Tyr Glu Glu	Ala Met Asn Val Ala	Leu		
	635		640		645
Lys Ala Leu Gly	Thr Lys Glu Gly Ile	Lys Glu Pro Glu Ser	Phe		
	650		655		660
Asn Ala Ala Val	Gln Glu Thr Glu Ala	Pro Tyr Ile Ser Ile	Ala		
	665		670		675
Cys Asp Leu Ile	Lys Glu Thr Lys Leu	Ser Thr Glu Pro Ser	Pro		
	680		685		690
Asp Phe Ser Asn	Tyr Ser Glu Ile Ala	Lys Phe Glu Lys Ser	Val		
	695		700		705
Pro Glu His Ala	Glu Leu Val Glu Asp	Ser Ser Pro Glu Ser	Glu		
	710		715		720
Pro Val Asp Leu	Phe Ser Asp Asp Ser	Ile Pro Glu Val Pro	Gln		
	725		730		735
Thr Gln Glu Glu	Ala Val Met Leu Met	Lys Glu Ser Leu Thr	Glu		
	740		745		750
Val Ser Glu Thr	Val Ala Gln His Lys	Glu Glu Arg Leu Ser	Ala		
	755		760		765
Ser Pro Gln Glu	Leu Gly Lys Pro Tyr	Leu Glu Ser Phe Gln	Pro		
	770		775		780
Asn Leu His Ser	Thr Lys Asp Ala Ala	Ser Asn Asp Ile Pro	Thr		
	785		790		795
Leu Thr Lys Lys	Glu Lys Ile Ser Leu	Gln Met Glu Glu Phe	Asn		
	800		805		810
Thr Ala Ile Tyr	Ser Asn Asp Asp Leu	Leu Ser Ser Lys Glu	Asp		
	815		820		825
Lys Ile Lys Glu	Ser Glu Thr Phe Ser	Asp Ser Ser Pro Ile	Glu		

	830		835		840
Ile Ile Asp Glu	Phe Pro Thr Phe Val	Ser Ala Lys Asp Asp Ser			
	845	850			855
Pro Lys Leu Ala	Lys Glu Tyr Thr Asp	Leu Glu Val Ser Asp Lys			
	860	865			870
Ser Glu Ile Ala	Asn Ile Gln Ser Gly	Ala Asp Ser Leu Pro Cys			
	875	880			885
Leu Glu Leu Pro	Cys Asp Leu Ser Phe	Lys Asn Ile Tyr Pro Lys			
	890	895			900
Asp Glu Val His	Val Ser Asp Glu Phe	Ser Glu Asn Arg Ser Ser			
	905	910			915
Val Ser Lys Ala	Ser Ile Ser Pro Ser	Asn Val Ser Ala Leu Glu			
	920	925			930
Pro Gln Thr Glu	Met Gly Ser Ile Val	Lys Ser Lys Ser Leu Thr			
	935	940			945
Lys Glu Ala Glu	Lys Lys Leu Pro Ser	Asp Thr Glu Lys Glu Asp			
	950	955			960
Arg Ser Leu Ser	Ala Val Leu Ser Ala	Glu Leu Ser Lys Thr Ser			
	965	970			975
Val Val Asp Leu	Leu Tyr Trp Arg Asp	Ile Lys Lys Thr Gly Val			
	980	985			990
Val Phe Gly Ala	Ser Leu Phe Leu Leu	Leu Ser Leu Thr Val Phe			
	995	1000			1005
Ser Ile Val Ser	Val Thr Ala Tyr Ile	Ala Leu Ala Leu Leu Ser			
	1010	1015			1020
Val Thr Ile Ser	Phe Arg Ile Tyr Lys	Gly Val Ile Gln Ala Ile			
	1030	1030			1035
Gln Lys Ser Asp	Glu Gly His Pro Phe	Arg Ala Tyr Leu Glu Ser			
	1040	1045			1050
Glu Val Ala Ile	Ser Glu Glu Leu Val	Gln Lys Tyr Ser Asn Ser			
	1055	1060			1065
Ala Leu Gly His	Val Asn Ser Thr Ile	Lys Glu Leu Arg Arg Leu			
	1070	1075			1080
Phe Leu Val Asp	Asp Leu Val Asp Ser	Leu Lys Phe Ala Val Leu			
	1085	1090			1095
Met Trp Val Phe	Thr Tyr Val Gly Ala	Leu Phe Asn Gly Leu Thr			
	1100	1105			1110
Leu Leu Ile Leu	Ala Leu Ile Ser Leu	Phe Ser Ile Pro Val Ile			
	1115	1120			1125
Tyr Glu Arg His	Gln Val Gln Ile Asp	His Tyr Leu Gly Leu Ala			
	1130	1135			1140
Asn Lys Ser Val	Lys Asp Ala Met Ala	Lys Ile Gln Ala Lys Ile			

1145

1150

1155

Pro Gly Leu Lys Arg Lys Ala Asp  
1160

&lt;210&gt; 2

&lt;211&gt; 1192

&lt;212&gt; PRT

&lt;213&gt; human

&lt;220&gt;

&lt;223&gt; human Nogo-A protein

&lt;400&gt; 2

Met	Glu	Asp	Leu	Asp	Gln	Ser	Pro	Leu	Val	Ser	Ser	Ser	Asp	Ser	1	5	10	15
Pro	Pro	Arg	Pro	Gln	Pro	Ala	Phe	Arg	Tyr	Gln	Phe	Val	Arg	Glu	20	25	30	
Pro	Glu	Asp	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Asp	Glu	35	40	45	
Asp	Glu	Asp	Leu	Glu	Glu	Leu	Glu	Val	Leu	Glu	Arg	Lys	Pro	Ala	50	55	60	
Ala	Gly	Leu	Ser	Ala	Ala	Pro	Val	Pro	Thr	Ala	Pro	Ala	Ala	Gly	65	70	75	
Ala	Pro	Leu	Met	Asp	Phe	Gly	Asn	Glu	Phe	Val	Pro	Pro	Ala	Pro	80	85	90	
Arg	Gly	Pro	Leu	Pro	Ala	Ala	Pro	Pro	Val	Ala	Pro	Glu	Arg	Gln	95	100	105	
Pro	Ser	Trp	Asp	Pro	Ser	Pro	Val	Ser	Ser	Thr	Val	Pro	Ala	Pro	110	115	120	
Ser	Pro	Leu	Ser	Ala	Ala	Ala	Val	Ser	Pro	Ser	Lys	Leu	Pro	Glu	125	130	135	
Asp	Asp	Glu	Pro	Pro	Ala	Arg	Pro	Pro	Pro	Pro	Pro	Pro	Ala	Ser	140	145	150	
Val	Ser	Pro	Gln	Ala	Glu	Pro	Val	Trp	Thr	Pro	Pro	Ala	Pro	Ala	155	160	165	
Pro	Ala	Ala	Pro	Pro	Ser	Thr	Pro	Ala	Ala	Pro	Lys	Arg	Arg	Gly	170	185	180	
Ser	Ser	Gly	Ser	Val	Asp	Glu	Thr	Leu	Phe	Ala	Leu	Pro	Ala	Ala	185	190	195	
Ser	Glu	Pro	Val	Ile	Arg	Ser	Ser	Ala	Glu	Asn	Met	Glu	Leu	Lys	200	205	210	
Glu	Gln	Pro	Gly	Asn	Thr	Ile	Ser	Ala	Gly	Gln	Glu	Asp	Phe	Pro	215	220	225	
Ser	Val	Leu	Leu	Glu	Thr	Ala	Ala	Ser	Leu	Pro	Ser	Leu	Ser	Pro				

				230					235					240
Leu	Ser	Ala	Ala	Ser	Phe	Lys	Glu	His	Glu	Tyr	Leu	Glu	Asn	Leu
				245					250					255
Ser	Thr	Val	Leu	Pro	Thr	Glu	Gly	Thr	Leu	Gln	Glu	Asn	Val	Ser
				260					265					270
Glu	Ala	Ser	Lys	Glu	Val	Ser	Glu	Lys	Ala	Lys	Thr	Leu	Leu	Ile
				275					280					285
Asp	Arg	Asp	Leu	Thr	Glu	Phe	Ser	Glu	Leu	Glu	Tyr	Ser	Glu	Met
				290					295					300
Gly	Ser	Ser	Phe	Ser	Val	Ser	Pro	Lys	Ala	Glu	Ser	Ala	Val	Ile
				305					310					315
Val	Ala	Asn	Pro	Arg	Glu	Glu	Ile	Ile	Val	Lys	Asn	Lys	Asp	Glu
				320					325					330
Glu	Glu	Lys	Leu	Val	Ser	Asn	Ans	Ile	Leu	His	Asn	Gln	Gln	Glu
				335					340					345
Leu	Pro	Thr	Ala	Leu	Thr	Lys	Leu	Val	Lys	Glu	Asp	Glu	Val	Val
				350					355					360
Ser	Ser	Glu	Lys	Ala	Lys	Asp	Ser	Phe	Asn	Glu	Lys	Arg	Val	Ala
				365					370					385
Val	Glu	Ala	Pro	Met	Arg	Glu	Glu	Tyr	Ala	Asp	Phe	Lys	Pro	Phe
				380					385					390
Glu	Arg	Val	Trp	Glu	Val	Lys	Asp	Ser	Lys	Glu	Asp	Ser	Asp	Met
				395					400					405
Leu	Ala	Ala	Gly	Gly	Lys	Ile	Glu	Ser	Asn	Leu	Glu	Ser	Lys	Val
				410					415					420
Asp	Lys	Lys	Cys	Phe	Ala	Asp	Ser	Leu	Glu	Gln	Thr	Asn	His	Glu
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Lys	Asn	Ser	Glu	Ser	Ser	Asn	Asp	Asp	Thr	Ser	Phe	Pro	Ser	Thr
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Pro	Glu	Gly	Ile	Lys	Asp	Arg	Pro	Gly	Ala	Tyr	Ile	Thr	Cys	Ala
				455					460					465
Pro	Phe	Asn	Pro	Ala	Ala	Thr	Glu	Ser	Ile	Ala	Thr	Asn	Ile	Phe
				470					475					480
Pro	Leu	Leu	Gly	Asp	Pro	Thr	Ser	Glu	Asn	Lys	Thr	Asp	Glu	Lys
				485					490					495
Lys	Ile	Glu	Glu	Lys	Lys	Ala	Gln	Ile	Val	Thr	Glu	Lys	Asn	Thr
				500					505					510
Ser	Thr	Lys	Thr	Ser	Asn	Pro	Phe	Leu	Val	Ala	Ala	Gln	Glu	Ser
				515					520					525
Glu	Thr	Asp	Tyr	Val	Thr	Thr	Asp	Asn	Leu	Thr	Lys	Val	Thr	Glu
				530					535					540
Glu	Val	Val	Ala	Asn	Met	Pro	Glu	Gly	Leu	Thr	Pro	Asp	Leu	Val



	545		550		555
Gln Glu Ala Cys	Glu Ser Glu Leu Asn	Glu Val Thr Gly Thr	Lys		
	560		565		570
Ile Ala Tyr Glu	Thr Lys Met Asp Leu	Val Gln Thr Ser Glu	Val		
	575		580		585
Met Gln Glu Ser	Leu Tyr Pro Ala Ala	Gln Leu Cys Pro Ser	Phe		
	590		595		600
Glu Glu Ser Glu	Ala Thr Pro Ser Pro	Val Leu Pro Asp Ile	Val		
	605		610		615
Met Glu Ala Pro	Leu Asn Ser Ala Val	Pro Ser Ala Gly Ala	Ser		
	620		625		630
Val Ile Gln Pro	Ser Ser Ser Pro Leu	Glu Ala Ser Ser Val	Gln		
	635		640		645
Tyr Glu Ser Ile	Lys His Glu Pro Glu	Asn Pro Pro Pro Tyr	Glu		
	650		655		660
Glu Ala Met Ser	Val Ser Leu Lys Lys	Val Ser Gly Ile Lys	Glu		
	665		670		675
Glu Ile Lys Glu	Pro Glu Asn Ile Asn	Ala Ala Leu Gln Glu	Thr		
	680		685		690
Glu Ala Pro Tyr	Ile Ser Ile Ala Cys	Asp Leu Ile Lys Glu	Thr		
	695		700		705
Lys Leu Ser Ala	Glu Pro Ala Pro Glu	Phe Ser Asp Tyr Ser	Glu		
	710		715		720
Met Ala Lys Val	Glu Gln Pro Val Pro	Asp His Ser Glu Leu	Val		
	725		730		735
Glu Asp Ser Ser	Pro Asp Ser Glu Pro	Val Asp Leu Phe Ser	Asp		
	740		745		750
Asp Ser Ile Pro	Asp Val Pro Gln Lys	Gln Asp Glu Thr Val	Met		
	755		760		765
Leu Val Lys Glu	Ser Leu Thr Glu Thr	Ser Phe Glu Ser Met	Ile		
	770		775		780
Glu Tyr Glu Gln	Lys Glu Lys Leu Ser	Ala Leu Pro Pro Glu	Gly		
	785		790		795
Gly Lys Pro Tyr	Leu Glu Ser Phe Lys	Leu Ser Leu Asp Asn	Thr		
	800		805		810
Lys Asp Thr Leu	Leu Pro Asp Glu Val	Ser Thr Leu Ser Lys	Lys		
	815		820		825
Glu Lys Ile Pro	Ile Gln Met Glu Glu	Leu Ser Thr Ala Val	Tyr		
	830		835		840
Ser Asn Asp Asp	Leu Phe Ile Ser Lys	Glu Ala Gln Ile Arg	Glu		
	845		850		855
Thr Glu Thr Phe	Ser Asp Ser Ser Pro	Ile Glu Ile Ile Asp	Glu		

	860		865		870
Phe Pro Thr Leu	Ile Ser Ser Lys Thr	Asp Ser Phe Ser Lys Leu			
	875	880			885
Ala Arg Glu Tyr	Thr Asp Leu Glu Val	Ser His Lys Ser Glu Ile			
	890	895			900
Ala Gln Ala Pro	Asp Gly Ala Gly Ser	Leu Pro Cys Thr Glu Leu			
	905	910			915
Pro His Asp Leu	Ser Leu Lys Asn Ile	Gln Pro Lys Val Glu Glu			
	920	925			930
Lys Ile Ser Phe	Ser Asp Asp Phe Ser	Lys Asn Gly Ser Ala Thr			
	935	940			945
Ser Lys Val Leu	Leu Leu Pro Pro Asp	Val Ser Ala Leu Ala Thr			
	950	955			960
Gln Ala Glu Ile	Glu Ser Ile Val Lys	Pro Lys Val Leu Val Lys			
	965	970			975
Glu Ala Glu Lys	Lys Leu Pro Ser Asp	Thr Glu Lys Glu Asp Arg			
	980	985			990
Ser Pro Ser Ala	Ile Phe Ser Ala Glu	Leu Ser Lys Thr Ser Val			
	995	1000			1005
Val Asp Leu Leu	Tyr Trp Arg Asp Ile	Lys Lys Thr Gly Val Val			
	1010	1015			1020
Phe Gly Ala Ser	Leu Phe Leu Leu Leu	Ser Leu Thr Val Phe Ser			
	1025	1030			1035
Ile Val Ser Val	Thr Ala Tyr Ile Ala	Leu Ala Leu Leu Ser Val			
	1040	1045			1050
Thr Ile Ser Phe	Arg Ile Tyr Lys Gly	Val Ile Gln Ala Ile Gln			
	1055	1060			1065
Lys Ser Asp Glu	Gly His Pro Phe Arg	Ala Tyr Leu Glu Ser Glu			
	1070	1075			1080
Val Ala Ile Ser	Glu Glu Leu Val Gln	Lys Tyr Ser Asn Ser Ala			
	1085	1090			1095
Leu Gly His Val	Asn Cys Thr Ile Lys	Glu Leu Arg Arg Leu Phe			
	1100	1105			1110
Leu Val Asp Asp	Leu Val Asp Ser Leu	Lys Phe Ala Val Leu Met			
	1115	1120			1125
Trp Val Phe Thr	Tyr Val Gly Ala Leu	Phe Asn Gly Leu Thr Leu			
	1130	1135			1140
Leu Ile Leu Ala	Leu Ile Ser Leu Phe	Ser Val Pro Val Ile Tyr			
	1145	1150			1155
Glu Arg His Gln	Ala Gln Ile Asp His	Tyr Leu Gly Leu Ala Asn			
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Lys Asn Val Lys	Asp Ala Met Ala Lys	Ile Gln Ala Lys Ile Pro			

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Gly Leu Lys Arg Lys Ala Glu		
1190		
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<223> k: g or t

<220>  
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<210> 9  
<211> 66  
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<220>  
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<223> n: a, g, c or t; m: a or c

<220>  
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<223> n: a, g, c or t; m: a or c

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<400> 9

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<223> n: a, g, c or t; m: a or c

<220>  
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<400> 10

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mmnnattttg acagtaatac gttgc 74

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<212> PRT  
<213> mouse

<220>  
<221>  
<222> (1) ... (121)  
<223> variable domain of the heavy chain of antibody IN-1

<400> 11

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Thr	Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Tyr	Thr	Phe	Thr	
				20					25					30	
Asn	Tyr	Trp	Leu	Gly	Trp	Val	Lys	Gln	Arg	Pro	Gly	His	Gly	Leu	
				35					40					45	
Glu	Trp	Ile	Gly	Asp	Ile	Tyr	Pro	Gly	Gly	Gly	Tyr	Thr	Asn	Tyr	
				50					55					60	
Asn	Glu	Lys	Phe	Lys	Gly	Lys	Ala	Thr	Leu	Thr	Ala	Asp	Thr	Ser	
				65					70					75	
Ser	Ser	Thr	Ala	Tyr	Met	Gln	Leu	Ser	Ser	Leu	Thr	Ser	Glu	Asp	
				80					85					90	
Ser	Ala	Val	Tyr	Phe	Cys	Ala	Arg	Phe	Tyr	Tyr	Gly	Ser	Ser	Tyr	
				95					100					105	
Trp	Tyr	Phe	Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	
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Ser

<210> 12  
<211> 107  
<212> PRT  
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<220>  
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<222> (1) ... (107)

<223> variable domain of the light chain of the antibody II.1.8

<400> 12

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Gly Glu Thr Val Thr Ile Thr Cys Gly Ala Ser Glu Asn Ile Tyr
             20             25             30
Gly Ala Leu Asn Trp Tyr Gln Arg Lys Gln Gly Lys Ser Pro Gln
             35             40             45
Leu Leu Ile Tyr Gly Ala Thr Asn Leu Ala Asp Gly Met Ser Ser
             50             55             60
Arg Phe Ser Gly Ser Gly Ser Gly Arg Gln Tyr Ser Leu Lys Ile
             65             70             75
Ser Ser Leu His Pro Asp Asp Val Ala Thr Tyr Tyr Cys Gln Asn
             80             85             90
Ile Asn Arg Val Pro Val Thr Phe Gly Ala Gly Thr Lys Leu Glu
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Ile Lys

<210> 13

<211> 2238

<212> DNA

<213> artifical sequence ..

<220>

<221> sig\_peptide

<222> (22)...(84)

<220>

<221> mat\_peptide

<222> (85)...(2238)

<223> fusion protein of truncated rat Nogo-A fragment and Strep-tag II

<220>

<221> CDS

<222> (85)...(2208)

<223> mature truncated Nogo-A

<220>

<221> CDS

<222> (2209)...(2238)

<223> Strep-tag II affinity tag

<400> 13

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                      Met Lys Lys Thr Ala Ile Ala Ile

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45

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gca	gtg	gca	ctg	gct	ggg	ttc	gct	acc	gta	gcg	cag	gcc	tct	ttt	90																
Ala	Val	Ala	Leu	Ala	Gly	Phe	Ala	Thr	Val	Ala	Gln	Ala	Ser	Phe																	
			-10						-5				-1		1																
aaa	gaa	cat	gga	tac	ctt	ggg	aac	tta	tca	gca	gtg	tca	tcc	tca	135																
Lys	Glu	His	Gly	Tyr	Leu	Gly	Asn	Leu	Ser	Ala	Val	Ser	Ser	Ser																	
			5			10						15																			
gaa	gga	aca	att	gaa	gaa	act	tta	aat	gaa	gct	tct	aaa	gag	ttg	180																
Glu	Gly	Thr	Ile	Glu	Glu	Thr	Leu	Asn	Glu	Ala	Ser	Lys	Glu	Leu																	
			20				25						30																		
cca	gag	agg	gca	aca	aat	cca	ttt	gta	aat	aga	gat	tta	gca	gaa	225																
Pro	Glu	Arg	Ala	Thr	Asn	Pro	Phe	Val	Asn	Arg	Asp	Leu	Ala	Glu																	
			35				40						45																		
ttt	tca	gaa	tta	gaa	tat	tca	gaa	atg	gga	tca	tct	ttt	aaa	ggc	270																
Phe	Ser	Glu	Leu	Glu	Tyr	Ser	Glu	Met	Gly	Ser	Ser	Phe	Lys	Gly																	
			50				55						60																		
tcc	cca	aaa	gga	gag	tca	gcc	ata	tta	gta	gaa	aac	act	aag	gaa	315																
Ser	Pro	Lys	Gly	Glu	Ser	Ala	Ile	Leu	Val	Glu	Asn	Thr	Lys	Glu																	
			65				70						75																		
gaa	gta	att	gtg	agg	agt	aaa	gac	aaa	gag	gat	tta	gtt	tgt	agt	360																
Glu	Val	Ile	Val	Arg	Ser	Lys	Asp	Lys	Glu	Asp	Leu	Val	Cys	Ser																	
			80				85						90																		
gca	gcc	ctt	cac	agt	cca	caa	gaa	tca	cct	gtg	ggg	aaa	gaa	gac	405																
Ala	Ala	Leu	His	Ser	Pro	Gln	Glu	Ser	Pro	Val	Gly	Lys	Glu	Asp																	
			95				100						105																		
aga	gtt	gtg	tct	cca	gaa	aag	aca	atg	gac	att	ttt	aat	gaa	atg	450																
Arg	Val	Val	Ser	Pro	Glu	Lys	Thr	Met	Asp	Ile	Phe	Asn	Glu	Met																	
			110				115						120																		
cag	atg	tca	gta	gta	gca	cct	gtg	agg	gaa	gag	tat	gca	gac	ttt	495																
Gln	Met	Ser	Val	Val	Ala	Pro	Val	Arg	Glu	Glu	Tyr	Ala	Asp	Phe																	
			125				130						135																		
aag	cca	ttt	gaa	caa	gca	tgg	gaa	gtg	aaa	gat	act	tat	gag	gga	540																
Lys	Pro	Phe	Glu	Gln	Ala	Trp	Glu	Val	Lys	Asp	Thr	Tyr	Glu	Gly																	
			140				145						150																		
agt	agg	gat	gtg	ctg	gct	gct	aga	gct	aat	gtg	gaa	agt	aaa	gtg	585																
Ser	Arg	Asp	Val	Leu	Ala	Ala	Arg	Ala	Asn	Val	Glu	Ser	Lys	Val																	
			155				160						165																		
gac	aga	aaa	tgc	ttg	gaa	gat	agc	ctg	gag	caa	aaa	agt	ctt	ggg	630																
Asp	Arg	Lys	Cys	Leu	Glu	Asp	Ser	Leu	Glu	Gln	Lys	Ser																			

Ser	Phe	Thr	Ser	Ala	Thr	Glu	Ser	Thr	Thr	Ala	Asn	Thr	Phe	Pro			
		215					220					225					
ttg	tta	gaa	gat	cat	act	tca	gaa	aat	aaa	aca	gat	gaa	aaa	aaa	810		
Leu	Leu	Glu	Asp	His	Thr	Ser	Glu	Asn	Lys	Thr	Asp	Glu	Lys	Lys			
		230					235					240					
ata	gaa	gaa	agg	aag	gcc	caa	att	ata	aca	gag	aag	act	agc	ccc	855		
Ile	Glu	Glu	Arg	Lys	Ala	Gln	Ile	Ile	Thr	Glu	Lys	Thr	Ser	Pro			
		245					250					255					
aaa	acg	tca	aat	cct	ttc	ctt	gta	gca	gta	cag	gat	tct	gag	gca	900		
Lys	Thr	Ser	Asn	Pro	Phe	Leu	Val	Ala	Val	Gln	Asp	Ser	Glu	Ala			
		260					265					270					
gat	tat	gtt	aca	aca	gat	acc	tta	tca	aag	gtg	act	gag	gca	gca	945		
Asp	Tyr	Val	Thr	Thr	Asp	Thr	Leu	Ser	Lys	Val	Thr	Glu	Ala	Ala			
		275					280					285					
gtg	tca	aac	atg	cct	gaa	ggt	ctg	acg	cca	gat	tta	gtt	cag	gaa	990		
Val	Ser	Asn	Met	Pro	Glu	Gly	Leu	Thr	Pro	Asp	Leu	Val	Gln	Glu			
		290					295					300					
gca	tgt	gaa	agt	gaa	ctg	aat	gaa	gcc	aca	ggt	aca	aag	att	gct	1035		
Ala	Cys	Glu	Ser	Glu	Leu	Asn	Glu	Ala	Thr	Gly	Thr	Lys	Ile	Ala			
		305					310					315					
tat	gaa	aca	aaa	gtg	gac	ttg	gtc	caa	aca	tca	gaa	gct	ata	caa	1080		
Tyr	Glu	Thr	Lys	Val	Asp	Leu	Val	Gln	Thr	Ser	Glu	Ala	Ile	Gln			
		320					325					330					
gaa	tca	ctt	tac	ccc	aca	gca	cag	ctt	tgc	cca	tca	ttt	gag	gaa	1125		
Glu	Ser	Leu	Tyr	Pro	Thr	Ala	Gln	Leu	Cys	Pro	Ser	Phe	Glu	Glu			
		335					340					345					
gct	gaa	gca	act	ccg	tca	cca	gtt	ttg	cct	gat	att	gtt	atg	gaa	1170		
Ala	Glu	Ala	Thr	Pro	Ser	Pro	Val	Leu	Pro	Asp	Ile	Val	Met	Glu			
		350					355					360					
gca	cca	tta	aat	tct	ctc	ctt	cca	agc	gct	ggt	gct	tct	gta	gtg	1215		
Ala	Pro	Leu	Asn	Ser	Leu	Leu	Pro	Ser	Ala	Gly	Ala	Ser	Val	Val			
		365					370					375					
cag	ccc	agt	gta	tcc	cca	ctg	gaa	gca	cct	cct	cca	gtt	agt	tat	1260		
Gln	Pro	Ser	Val	Ser	Pro	Leu	Glu	Ala	Pro	Pro	Pro	Val	Ser	Tyr			
		380					385					390					
gac	agt	ata	aag	ctt	gag	cct	gaa	aac	ccc	cca	cca	tat	gaa	gaa	1305		
Asp	Ser	Ile	Lys	Leu	Glu	Pro	Glu	Asn	Pro	Pro	Pro	Tyr	Glu	Glu			
		395					400					405					
gcc	atg	aat	gta	gca	cta	aaa	gct	ttg	gga	aca	aag	gaa	gga	ata	1350		
Ala	Met	Asn	Val	Ala	Leu	Lys	Ala	Leu	Gly	Thr	Lys	Glu	Gly	Ile			
		410					415					420					
aaa	gag	cct	gaa	agt	ttt	aat	gca	gct	gtt	cag	gaa	aca	gaa	gct	1395		
Lys	Glu	Pro	Glu	Ser	Phe	Asn	Ala	Ala	Val	Gln	Glu	Thr	Glu	Ala			
		425					430					435					
cct	tat	ata	tcc	att	gcg	tgt	gat	tta	att	aaa	gaa	aca	aag	ctc	1440		
Pro	Tyr	Ile	Ser	Ile	Ala	Cys	Asp	Leu	Ile	Lys	Glu	Thr	Lys	Leu			
		440					445					450					



tcc act gag cca agt cca gat ttc tct aat tat tca gaa ata gca	1485
Ser Thr Glu Pro Ser Pro Asp Phe Ser Asn Tyr Ser Glu Ile Ala	
455 460 465	
aaa ttc gag aag tcg gtg ccc gaa cac gct gag cta gtg gag gat	1530
Lys Phe Glu Lys Ser Val Pro Glu His Ala Glu Leu Val Glu Asp	
470 475 480	
tcc tca cct gaa tct gaa cca gtt gac tta ttt agt gat gat tcg	1575
Ser Ser Pro Glu Ser Glu Pro Val Asp Leu Phe Ser Asp Asp Ser	
485 490 495	
att cct gaa gtc cca caa aca caa gag gag gct gtg atg ctc atg	1620
Ile Pro Glu Val Pro Gln Thr Gln Glu Glu Ala Val Met Leu Met	
500 505 510	
aag gag agt ctc act gaa gtg tct gag aca gta gcc cag cac aaa	1665
Lys Glu Ser Leu Thr Glu Val Ser Glu Thr Val Ala Gln His Lys	
515 520 525	
gag gag aga ctt agt gcc tca cct cag gag cta gga aag cca tat	1710
Glu Glu Arg Leu Ser Ala Ser Pro Gln Glu Leu Gly Lys Pro Tyr	
530 535 540	
tta gag tct ttt cag ccc aat tta cat agt aca aaa gat gct gca	1755
Leu Glu Ser Phe Gln Pro Asn Leu His Ser Thr Lys Asp Ala Ala	
545 550 555	
tct aat gac att cca aca ttg acc aaa aag gag aaa att tct ttg	1800
Ser Asn Asp Ile Pro Thr Leu Thr Lys Lys Glu Lys Ile Ser Leu	
560 565 570	
caa atg gaa gag ttt aat act gca att tat tca aat gat gac tta	1845
Gln Met Glu Glu Phe Asn Thr Ala Ile Tyr Ser Asn Asp Asp Leu	
575 580 585	
ctt tct tct aag gaa gac aaa ata aaa gaa agt gaa aca ttt tca	1890
Leu Ser Ser Lys Glu Asp Lys Ile Lys Glu Ser Glu Thr Phe Ser	
590 595 600	
gat tca tct ccg att gag ata ata gat gaa ttt ccc acg ttt gtc	1935
Asp Ser Ser Pro Ile Glu Ile Ile Asp Glu Phe Pro Thr Phe Val	
605 610 615	
agt gct aaa gat gat tct cct aaa tta gcc aag gag tac act gat	1980
Ser Ala Lys Asp Asp Ser Pro Lys Leu Ala Lys Glu Tyr Thr Asp	
620 625 630	
cta gaa gta tcc gac aaa agt gaa att gct aat atc caa agc ggg	2025
Leu Glu Val Ser Asp Lys Ser Glu Ile Ala Asn Ile Gln Ser Gly	
635 640 645	
gca gat tca ttg cct tgc tta gaa ttg ccc tgt gac ctt tct ttc	2070
Ala Asp Ser Leu Pro Cys Leu Glu Leu Pro Cys Asp Leu Ser Phe	
650 655 660	
aag aat ata tat cct aaa gat gaa gta cat gtt tca gat gaa ttc	2115
Lys Asn Ile Tyr Pro Lys Asp Glu Val His Val Ser Asp Glu Phe	
665 670 675	
tcc gaa aat agg tcc agt gta tct aag gca tcc ata tcg cct tca	2160
Ser Glu Asn Arg Ser Ser Val Ser Lys Ala Ser Ile Ser Pro Ser	
680 685 690	

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aat gtc tct gct ttg gaa cct cag aca gaa atg ggc agc ata gtt 2205
Asn Val Ser Ala Leu Glu Pro Gln Thr Glu Met Gly Ser Ile Val
      695                      700                      705

```

```

aaa agc gct tgg cgt cac ccg cag ttc ggt ggt taa taa gctt 2248
Lys Ser Ala Trp Arg His Pro Gln Phe Gly Gly End
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<210> 14
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<212> DNA
<213> artifical sequence

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<220>
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<222> (22)...(84)

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<220>
<221> mat_peptide
<222> (85)...(2460)
<223> fusion protein of truncated rat Nogo-A fragment and Strep-
tag II

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<220>
<221> CDS
<222> (85)...(2430)
<223> mature truncated Nogo-A

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<220>
<221> CDS
<222> (2431)...(2460)
<223> Strep-tag II affinity tag

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<400> 14

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tctagataac gagggcaaaa a atg aaa aag aca gct atc gcg att 45
                Met Lys Lys Thr Ala Ile Ala Ile
                -21 -20                      -15

```

```

gca gtg gca ctg gct ggt ttc gct acc gta gcg cag gcc gag acc 90
Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Glu Thr
      -10                      -5                      -1 1

```

```

gct ctt cct gct gca tct gag cct gtg ata ccc tcc tct gca gaa 135
Ala Leu Pro Ala Ala Ser Glu Pro Val Ile Pro Ser Ser Ala Glu
      5                      10                      15

```

```

ctt ttt aaa att atg gat ttg atg gag cag cca ggt aac act gtt 225
Leu Phe Lys Ile Met Asp Leu Met Glu Gln Pro Gly Asn Thr Val
      20                      25                      30

```

```

tcg tct ggt caa gag gat ttc cca tct gtc ctg ctt gaa act gct 270
Ser Ser Gly Gln Glu Asp Phe Pro Ser Val Leu Leu Glu Thr Ala
      35                      40                      45

```

```

gcc tct ctt cct tct cta tct cct ctc tca act gtt tct ttt aaa 315
Ala Ser Leu Pro Ser Leu Ser Pro Leu Ser Thr Val Ser Phe Lys

```

50	55	60	
gaa cat gga tac ctt ggt aac tta tca gca gtg tca tcc tca gaa	360		
Glu His Gly Tyr Leu Gly Asn Leu Ser Ala Val Ser Ser Ser Glu			
65	70	75	
gga aca att gaa gaa act tta aat gaa gct tct aaa gag ttg cca	405		
Gly Thr Ile Glu Glu Thr Leu Asn Glu Ala Ser Lys Glu Leu Pro			
80	85	90	
gag agg gca aca aat cca ttt gta aat aga gat tta gca gaa ttt	450		
Glu Arg Ala Thr Asn Pro Phe Val Asn Arg Asp Leu Ala Glu Phe			
95	100	105	
tca gaa tta gaa tat tca gaa atg gga tca tct ttt aaa ggc tcc	495		
Ser Glu Leu Glu Tyr Ser Glu Met Gly Ser Ser Phe Lys Gly Ser			
110	115	120	
cca aaa gga gag tca gcc ata tta gta gaa aac act aag gaa gaa	540		
Pro Lys Gly Glu Ser Ala Ile Leu Val Glu Asn Thr Lys Glu Glu			
125	130	135	
gta att gtg agg agt aaa gac aaa gag gat tta gtt tgt agt gca	585		
Val Ile Val Arg Ser Lys Asp Lys Glu Asp Leu Val Cys Ser Ala			
140	145	150	
gcc ctt cac agt cca caa gaa tca cct gtg ggt aaa gaa gac aga	630		
Ala Leu His Ser Pro Gln Glu Ser Pro Val Gly Lys Glu Asp Arg			
155	160	165	
gtt gtg tct cca gaa aag aca atg gac att ttt aat gaa atg cag	675		
Val Val Ser Pro Glu Lys Thr Met Asp Ile Phe Asn Glu Met Gln			
170	175	180	
atg tca gta gta gca cct gtg agg gaa gag tat gca gac ttt aag	720		
Met Ser Val Val Ala Pro Val Arg Glu Glu Tyr Ala Asp Phe Lys			
185	190	195	
cca ttt gaa caa gca tgg gaa gtg aaa gat act tat gag gga agt	765		
Pro Phe Glu Gln Ala Trp Glu Val Lys Asp Thr Tyr Glu Gly Ser			
200	205	210	
agg gat gtg ctg gct gct aga gct aat gtg gaa agt aaa gtg gac	810		
Arg Asp Val Leu Ala Ala Arg Ala Asn Val Glu Ser Lys Val Asp			
215	220	225	
aga aaa tgc ttg gaa gat agc ctg gag caa aaa agt ctt ggg aag	855		
Arg Lys Cys Leu Glu Asp Ser Leu Glu Gln Lys Ser Leu Gly Lys			
230	235	240	
gat agt gaa ggc aga aat gag gat gct tct ttc ccc agt acc cca	900		
Asp Ser Glu Gly Arg Asn Glu Asp Ala Ser Phe Pro Ser Thr Pro			
245	250	255	
gaa cct gtg aag gac agc tcc aga gca tat att acc tgt gct tcc	945		
Glu Pro Val Lys Asp Ser Ser Arg Ala Tyr Ile Thr Cys Ala Ser			
260	265	270	
ttt acc tca gca acc gaa agc acc aca gca aac act ttc cct ttg	990		
Phe Thr Ser Ala Thr Glu Ser Thr Thr Ala Asn Thr Phe Pro Leu			
275	280	285	
tta gaa gat cat act tca gaa aat aaa aca gat gaa aaa aaa ata	1035		

Leu	Glu	Asp	His	Thr	Ser	Glu	Asn	Lys	Thr	Asp	Glu	Lys	Lys	Ile			
		290					295					300					
gaa	gaa	agg	aag	gcc	caa	att	ata	aca	gag	aag	act	agc	ccc	aaa	1080		
Glu	Glu	Arg	Lys	Ala	Gln	Ile	Ile	Thr	Glu	Lys	Thr	Ser	Pro	Lys			
		305					310					315					
acg	tca	aat	cct	ttc	ctt	gta	gca	gta	cag	gat	tct	gag	gca	gat	1125		
Thr	Ser	Asn	Pro	Phe	Leu	Val	Ala	Val	Gln	Asp	Ser	Glu	Ala	Asp			
		320					325					330					
tat	gtt	aca	aca	gat	acc	tta	tca	aag	gtg	act	gag	gca	gca	gtg	1170		
Tyr	Val	Thr	Thr	Asp	Thr	Leu	Ser	Lys	Val	Thr	Glu	Ala	Ala	Val			
		335					340					345					
tca	aac	atg	cct	gaa	ggg	ctg	acg	cca	gat	tta	gtt	cag	gaa	gca	1215		
Ser	Asn	Met	Pro	Glu	Gly	Leu	Thr	Pro	Asp	Leu	Val	Gln	Glu	Ala			
		350					355					360					
tgt	gaa	agt	gaa	ctg	aat	gaa	gcc	aca	ggg	aca	aag	att	gct	tat	1260		
Cys	Glu	Ser	Glu	Leu	Asn	Glu	Ala	Thr	Gly	Thr	Lys	Ile	Ala	Tyr			
		365					370					375					
gaa	aca	aaa	gtg	gac	ttg	gtc	caa	aca	tca	gaa	gct	ata	caa	gaa	1305		
Glu	Thr	Lys	Val	Asp	Leu	Val	Gln	Thr	Ser	Glu	Ala	Ile	Gln	Glu			
		380					385					390					
tca	ctt	tac	ccc	aca	gca	cag	ctt	tgc	cca	tca	ttt	gag	gaa	gct	1350		
Ser	Leu	Tyr	Pro	Thr	Ala	Gln	Leu	Cys	Pro	Ser	Phe	Glu	Glu	Ala			
		395					400					405					
gaa	gca	act	ccg	tca	cca	gtt	ttg	cct	gat	att	gtt	atg	gaa	gca	1395		
Glu	Ala	Thr	Pro	Ser	Pro	Val	Leu	Pro	Asp	Ile	Val	Met	Glu	Ala			
		410					415					420					
cca	tta	aat	tct	ctc	ctt	cca	agc	gct	ggg	gct	tct	gta	gtg	cag	1440		
Pro	Leu	Asn	Ser	Leu	Leu	Pro	Ser	Ala	Gly	Ala	Ser	Val	Val	Gln			
		425					430					435					
ccc	agt	gta	tcc	cca	ctg	gaa	gca	cct	cct	cca	gtt	agt	tat	gac	1485		
Pro	Ser	Val	Ser	Pro	Leu	Glu	Ala	Pro	Pro	Pro	Val	Ser	Tyr	Asp			
		440					445					450					
agt	ata	aag	ctt	gag	cct	gaa	aac	ccc	cca	cca	tat	gaa	gaa	gcc	1530		
Ser	Ile	Lys	Leu	Glu	Pro	Glu	Asn	Pro	Pro	Pro	Tyr	Glu	Glu	Ala			
		455					460					465					
atg	aat	gta	gca	cta	aaa	gct	ttg	gga	aca	aag	gaa	gga	ata	aaa	1575		
Met	Asn	Val	Ala	Leu	Lys	Ala	Leu	Gly	Thr	Lys	Glu	Gly	Ile	Lys			
		470					475					480					
gag	cct	gaa	agt	ttt	aat	gca	gct	gtt	cag	gaa	aca	gaa	gct	cct	1620		
Glu	Pro	Glu	Ser	Phe	Asn	Ala	Ala	Val	Gln	Glu	Thr	Glu	Ala	Pro			
		485					490					495					
tat	ata	tcc	att	gcg	tgt	gat	tta	att	aaa	gaa	aca	aag	ctc	tcc	1665		
Tyr	Ile	Ser	Ile	Ala	Cys	Asp	Leu	Ile	Lys	Glu	Thr	Lys	Leu	Ser			
		500					505					510					
act	gag	cca	agt	cca	gat	ttc	tct	aat	tat	tca	gaa	ata	gca	aaa	1710		
Thr	Glu	Pro	Ser	Pro	Asp	Phe	Ser	Asn	Tyr	Ser	Glu	Ile	Ala	Lys			
		515					520					525					

ttc gag aag tcg gtg ccc gaa cac gct gag cta gtg gag gat tcc	1755
Phe Glu Lys Ser Val Pro Glu His Ala Glu Leu Val Glu Asp Ser	
530 535 540	
tca cct gaa tct gaa cca gtt gac tta ttt agt gat gat tcg att	1800
Ser Pro Glu Ser Glu Pro Val Asp Leu Phe Ser Asp Asp Ser Ile	
545 550 555	
cct gaa gtc cca caa aca caa gag gag gct gtg atg ctc atg aag	1845
Pro Glu Val Pro Gln Thr Gln Glu Glu Ala Val Met Leu Met Lys	
560 565 570	
gag agt ctc act gaa gtg tct gag aca gta gcc cag cac aaa gag	1890
Glu Ser Leu Thr Glu Val Ser Glu Thr Val Ala Gln His Lys Glu	
575 580 585	
gag aga ctt agt gcc tca cct cag gag cta gga aag cca tat tta	1935
Glu Arg Leu Ser Ala Ser Pro Gln Glu Leu Gly Lys Pro Tyr Leu	
590 595 600	
gag tct ttt cag ccc aat tta cat agt aca aaa gat gct gca tct	1980
Glu Ser Phe Gln Pro Asn Leu His Ser Thr Lys Asp Ala Ala Ser	
605 610 615	
aat gac att cca aca ttg acc aaa aag gag aaa att tct ttg caa	2025
Asn Asp Ile Pro Thr Leu Thr Lys Lys Glu Lys Ile Ser Leu Gln	
620 625 630	
atg gaa gag ttt aat act gca att tat tca aat gat gac tta ctt	2070
Met Glu Glu Phe Asn Thr Ala Ile Tyr Ser Asn Asp Asp Leu Leu	
635 640 645	
tct tct aag gaa gac aaa ata aaa gaa agt gaa aca ttt tca gat	2115
Ser Ser Lys Glu Asp Lys Ile Lys Glu Ser Glu Thr Phe Ser Asp	
650 655 660	
tca tct ccg att gag ata ata gat gaa ttt ccc acg ttt gtc agt	2160
Ser Ser Pro Ile Glu Ile Ile Asp Glu Phe Pro Thr Phe Val Ser	
665 670 675	
gct aaa gat gat tct cct aaa tta gcc aag gag tac act gat cta	2205
Ala Lys Asp Asp Ser Pro Lys Leu Ala Lys Glu Tyr Thr Asp Leu	
680 685 690	
gaa gta tcc gac aaa agt gaa att gct aat atc caa agc ggg gca	2250
Glu Val Ser Asp Lys Ser Glu Ile Ala Asn Ile Gln Ser Gly Ala	
695 700 705	
gat tca ttg cct tgc tta gaa ttg ccc tgt gac ctt tct ttc aag	2295
Asp Ser Leu Pro Cys Leu Glu Leu Pro Cys Asp Leu Ser Phe Lys	
710 715 720	
aat ata tat cct aaa gat gaa gta cat gtt tca gat gaa ttc tcc	2340
Asn Ile Tyr Pro Lys Asp Glu Val His Val Ser Asp Glu Phe Ser	
725 730 735	
gaa aat agg tcc agt gta tct aag gca tcc ata tcg cct tca aat	2385
Glu Asn Arg Ser Ser Val Ser Lys Ala Ser Ile Ser Pro Ser Asn	
740 745 750	
gtc tct gct ttg gaa cct cag aca gaa atg ggc agc ata gtt aaa	2430
Val Ser Ala Leu Glu Pro Gln Thr Glu Met Gly Ser Ile Val Lys	
755 760 765	

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agc gct tgg cgt cac ccg cag ttc ggt ggt taa taa gctt      2470
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      770                      775

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<210> 15
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<222> (22)...(84)

<220>
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<222> (85)...(2271)
<223> fusion protein of Strep-tag II, truncated rat Nogo-A
fragment and hexahistidine tag

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<220>
<221> CDS
<222> (85)...(120)
<223> Strep-tag II affinity tag

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<220>
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<222> (121)...(2250)
<223> mature truncated Nogo-A

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<220>
<221> CDS
<222> (2251)...(2271)
<223> hexahistidine tag affinity tag

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<400> 15

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tctagataac gagggcaaaa a atg aaa aag aca gct atc gcg att      45
                Met Lys Lys Thr Ala Ile Ala Ile
                -21 -20                      -15

```

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gca gtg gca ctg gct ggt ttc gct acc gta gcg cag gcc gct agc  90
Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Ala Ser
                -10                      -5                      -1

```

```

tgg agc cac ccg cag ttc gaa aaa ggc gcc tct ttt aaa gaa cat 135
Trp Ser His Pro Gln Phe Glu Lys Gly Ala Ser Phe Lys Glu His
                5                      10                      15

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```

gga tac ctt ggt aac tta tca gca gtg tca tcc tca gaa gga aca 180
Gly Tyr Leu Gly Asn Leu Ser Ala Val Ser Ser Ser Glu Gly Thr
                20                      25                      30

```

```

att gaa gaa act tta aat gaa gct tct aaa gag ttg cca gag agg 225
Ile Glu Glu Thr Leu Asn Glu Ala Ser Lys Glu Leu Pro Glu Arg
                35                      40                      45

```

```

gca aca aat cca ttt gta aat aga gat tta gca gaa ttt tca gaa 270

```

Ala Thr Asn Pro Phe Val Asn Arg Asp Leu Ala Glu Phe Ser Glu	50	55	60	
tta gaa tat tca gaa atg gga tca tct ttt aaa ggc tcc cca aaa	315			
Leu Glu Tyr Ser Glu Met Gly Ser Ser Phe Lys Gly Ser Pro Lys	65	70	75	
gga gag tca gcc ata tta gta gaa aac act aag gaa gaa gta att	360			
Gly Glu Ser Ala Ile Leu Val Glu Asn Thr Lys Glu Glu Val Ile	80	85	90	
gtg agg agt aaa gac aaa gag gat tta gtt tgt agt gca gcc ctt	405			
Val Arg Ser Lys Asp Lys Glu Asp Leu Val Cys Ser Ala Ala Leu	95	100	105	
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His Ser Pro Gln Glu Ser Pro Val Gly Lys Glu Asp Arg Val Val	110	115	120	
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Ser Pro Glu Lys Thr Met Asp Ile Phe Asn Glu Met Gln Met Ser	125	130	135	
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Val Val Ala Pro Val Arg Glu Glu Tyr Ala Asp Phe Lys Pro Phe	140	145	150	
gaa caa gca tgg gaa gtg aaa gat act tat gag gga agt agg gat	585			
Glu Gln Ala Trp Glu Val Lys Asp Thr Tyr Glu Gly Ser Arg Asp	155	160	165	
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Val Leu Ala Ala Arg Ala Asn Val Glu Ser Lys Val Asp Arg Lys	170	175	180	
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Cys Leu Glu Asp Ser Leu Glu Gln Lys Ser Leu Gly Lys Asp Ser	185	190	195	
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Glu Gly Arg Asn Glu Asp Ala Ser Phe Pro Ser Thr Pro Glu Pro	200	205	210	
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Val Lys Asp Ser Ser Arg Ala Tyr Ile Thr Cys Ala Ser Phe Thr	215	220	225	
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Ser Ala Thr Glu Ser Thr Thr Ala Asn Thr Phe Pro Leu Leu Glu	230	235	240	
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Asp His Thr Ser Glu Asn Lys Thr Asp Glu Lys Lys Ile Glu Glu	245	250	255	
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Arg Lys Ala Gln Ile Ile Thr Glu Lys Thr Ser Pro Lys Thr Ser	260	265	270	
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Asn Pro Phe Leu Val Ala Val Gln Asp Ser Glu Ala Asp Tyr Val	275	280	285	

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Gln	Met	Ser 125	Val	Val	Ala	Pro	Val 130	Arg	Glu	Glu	Tyr	Ala 135	Asp	Phe			
Lys	Pro	Phe 140	Glu	Gln	Ala	Trp	Glu 145	Val	Lys	Asp	Thr	Tyr 150	Glu	Gly			

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Ile	Pro	Glu	Val	Pro	Gln	Thr	Gln	Glu	Glu	Ala	Val	Met	Leu	Met	500	505	510
Lys	Glu	Ser	Leu	Thr	Glu	Val	Ser	Glu	Thr	Val	Ala	Gln	His	Lys	515	520	525
Glu	Glu	Arg	Leu	Ser	Ala	Ser	Pro	Gln	Glu	Leu	Gly	Lys	Pro	Tyr	530	535	540
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Gln	Met	Glu	Glu	Phe	Asn	Thr	Ala	Ile	Tyr	Ser	Asn	Asp	Asp	Leu	575	580	585
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Lys	Ser	Ala	Trp	Arg	His	Pro	Gln	Phe	Gly	Gly					710	715	

**Claims**

1. An isolated truncated Nogo-A polypeptide that corresponds to a truncated form of the Nogo-A protein consisting of the amino acids 174 to 940 of the full length protein of rat Nogo-A (SEQ ID NO: 1, 1163 amino acids) or of the amino acids 246 to 966 of the human full length protein (SEQ ID NO: 2, 1192 amino acids).

2. The polypeptide of claim 1, wherein said truncated form of the Nogo-A protein consists of the amino acids 223 to 940 of the full length protein of rat Nogo-A.

3. The polypeptide of claim 1 or 2, wherein said truncated form is a polypeptide that begins with an amino acid residue selected from the amino acids 174 to 233 and that ends at a residue selected from amino acids 890 to 940 of the full length protein of rat Nogo-A.

4. A polypeptide selected from the group consisting of:

a) the polypeptide having the amino acid sequence consisting of amino acid residues 174 to 940 of the full length rat Nogo-A protein (SEQ ID NO: 1);

b) the polypeptide having the amino acid sequence consisting of amino acid residues 233 to 940 of the full length rat Nogo-A protein (SEQ ID NO:1);

c) the polypeptide having the amino acid sequence consisting of amino acid residues 246 to 966 of the full length human Nogo-A protein (SEQ ID NO:2);

d) a polypeptide having at least 50 % sequence identity to any of the polypeptides a) to c) wherein a fragment of the human Nogo-A protein consisting of amino acids 1 to 1024 is excluded;

e) a fragment of any of the polypeptides a) to d) wherein the fragment consisting of amino acids 624 to 639 of full length rat Nogo-A protein is excluded.

5. A fusion protein consisting of a Nogo-A polypeptide of any of the foregoing claims and a fusion partner fused to the N- and/or the C-terminus of the Nogo-A polypeptide.

6. The fusion protein of claim 5, wherein the fusion partner is a protein, a protein domain or a peptide.

7. A nucleic acid molecule encoding a polypeptide of any of claims 1 to 4 or a fusion protein of any of claims 5 or 6.



8. The nucleic acid molecule of claim 7 comprising the nucleotide sequence of positions 522 to 2822 or of positions 699 to 2822 of the coding sequence of rat Nogo-A deposited under accession number AJ242961 in the EMBL database.

5

9. A vector comprising a nucleic acid molecule of claim 7 or 8.

10. A host cell comprising a vector as defined in claim 9.

10

11. A method for the production of a Nogo-A polypeptide of any of claims 1 to 4 or a fusion protein of claims 5 or 6, wherein the Nogo-A polypeptide or the fusion protein of the Nogo-A polypeptide is produced starting from the nucleic acid coding for the Nogo-A polypeptide by means of an in vitro transcription and translation system and is isolated from this in vitro system or by means of genetic engineering methods in a bacterial or eucaryotic host organism and is isolated from this host organism or its culture.

15

12. The method of claim 11, wherein the Nogo-A polypeptide or fusion protein is produced by periplasmic expression in a bacterial host.

20

13. A method for identifying a compound having detectable affinity to a Nogo-A protein, comprising the steps of:

(a) contacting a truncated Nogo-A polypeptide or a fusion protein thereof as defined in any of claims 1 to 7 with a compound of interest under conditions that allow formation of a complex between the truncated Nogo-A protein and said compound; and

25

(b) detecting complex formation by means of a suitable signaling method.

14. The method of claim 13, wherein the compound of interest protein is an organic molecule, a peptide, a polypeptide or a nucleic acid.

30

15. The method of claim 14, wherein the polypeptide, the peptide or the nucleic acid is subjected to mutagenesis before contacting it with said truncated Nogo-A protein in step a).

35

16. The method of any of claims 13 to 15, wherein the polypeptide is selected from the group consisting of antibodies and muteins based on a polypeptide of the lipocalin family.

17. The method of claim 18, wherein the antibody is a mutein derived from the antibody IN-1 or a fragment or fusion protein thereof.

5 18. The method of any of claims 13 to 18, wherein the compound having binding affinity to a Nogo-A protein has a neutralizing effect on the neurite-growth-inhibiting activity of Nogo-A.

19. A method for identifying a compound having detectable affinity to a Nogo-A protein comprising the steps of:

10 (a) contacting a truncated Nogo-A polypeptide or a fusion protein thereof as defined in any of claims 1 to 7 with a plurality of compounds of interest under conditions that allow formation of a complex between the truncated Nogo-A protein and said compounds; and

15 (b) enriching at least one compound of interest that has detectable binding affinity to the Nogo-A protein by screening or selection and/or isolating said at least one compound.

20 20. The method of claim 19, wherein the plurality of compounds of interest are peptides, a polypeptides or nucleic acids that have been subjected to mutagenesis before contacting it with said truncated Nogo-A protein in step a).

21. An antibody or an fragment thereof having the variable domains of SEQ ID NO: 11 and SEQ ID NO: 12.

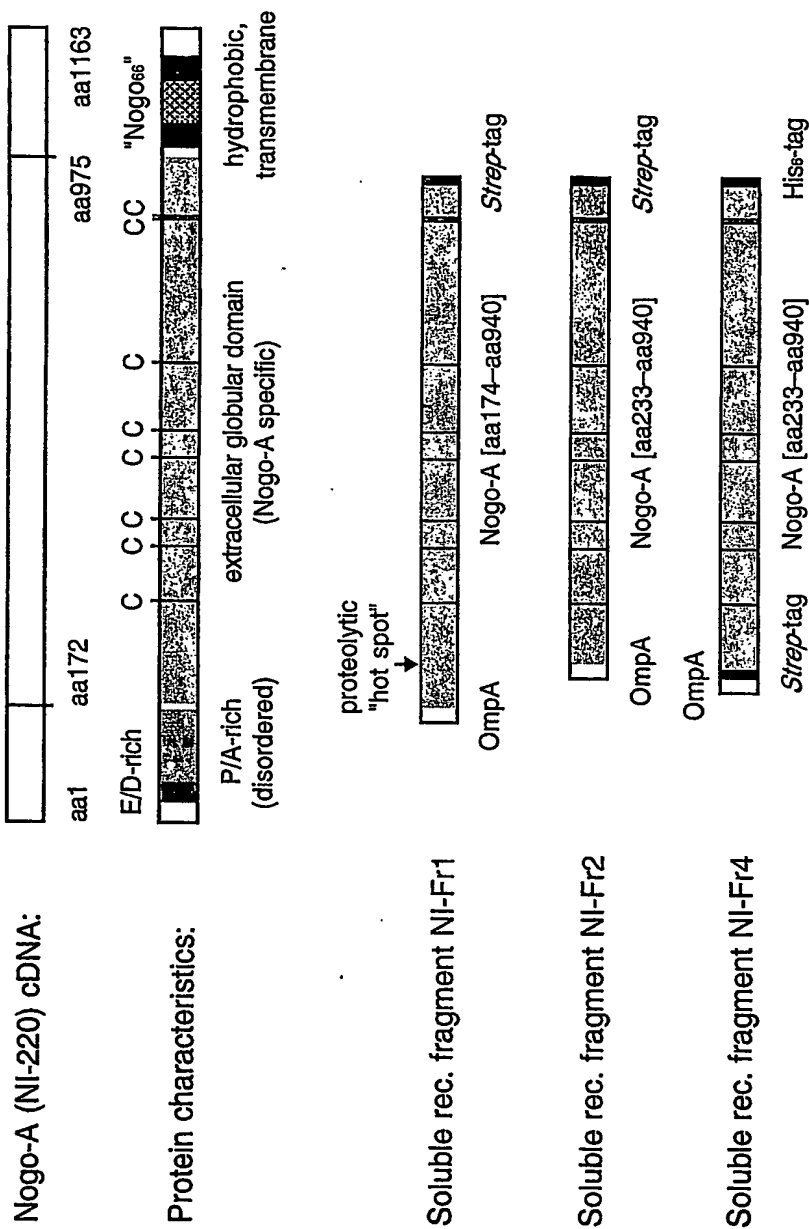


Fig. 1A

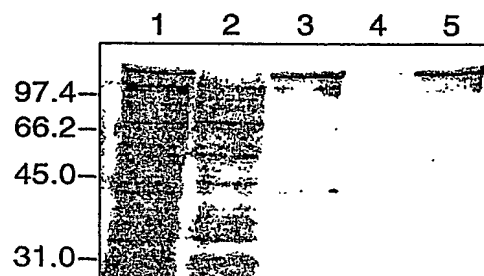


Fig. 1B

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IN-1  
A32F

CDR-L1  
1 24 34 40  
DIELTQSPAIMAASVGETVTITCGASENIYGALN~~WYQRKQ~~  
F

CDR-L2  
41 50 56 80  
GKSPQLLIYGATNLADGMSSRFSGSGSGRQYSLKISSLHP

CDR-L3  
81 89 97 107  
DDVATYYCONVLSTPRIFGAGTKLEIK  
RV C  
RV V  
INRV V

I.2.6  
I.2.6 (L96V)  
II.1.8

Fig. 2A

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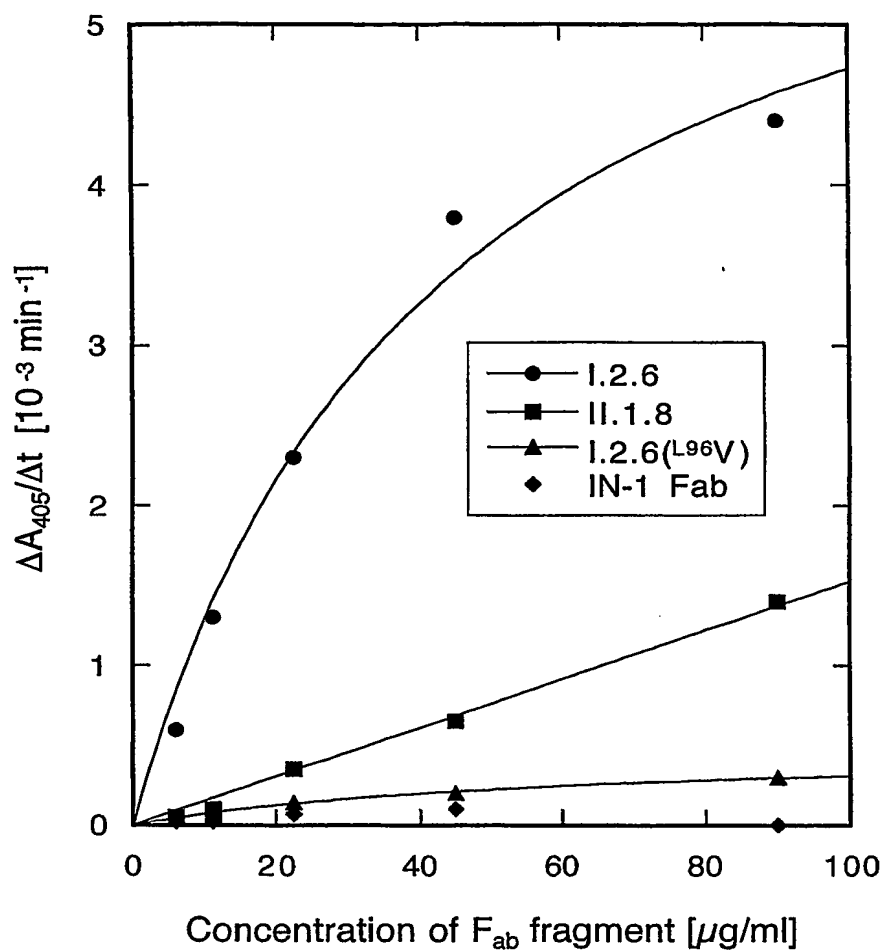


Fig. 2B

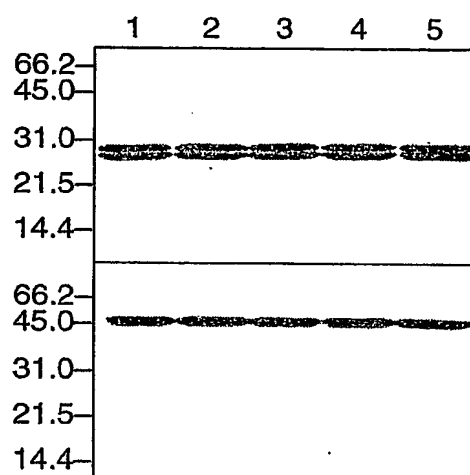


Fig. 3A

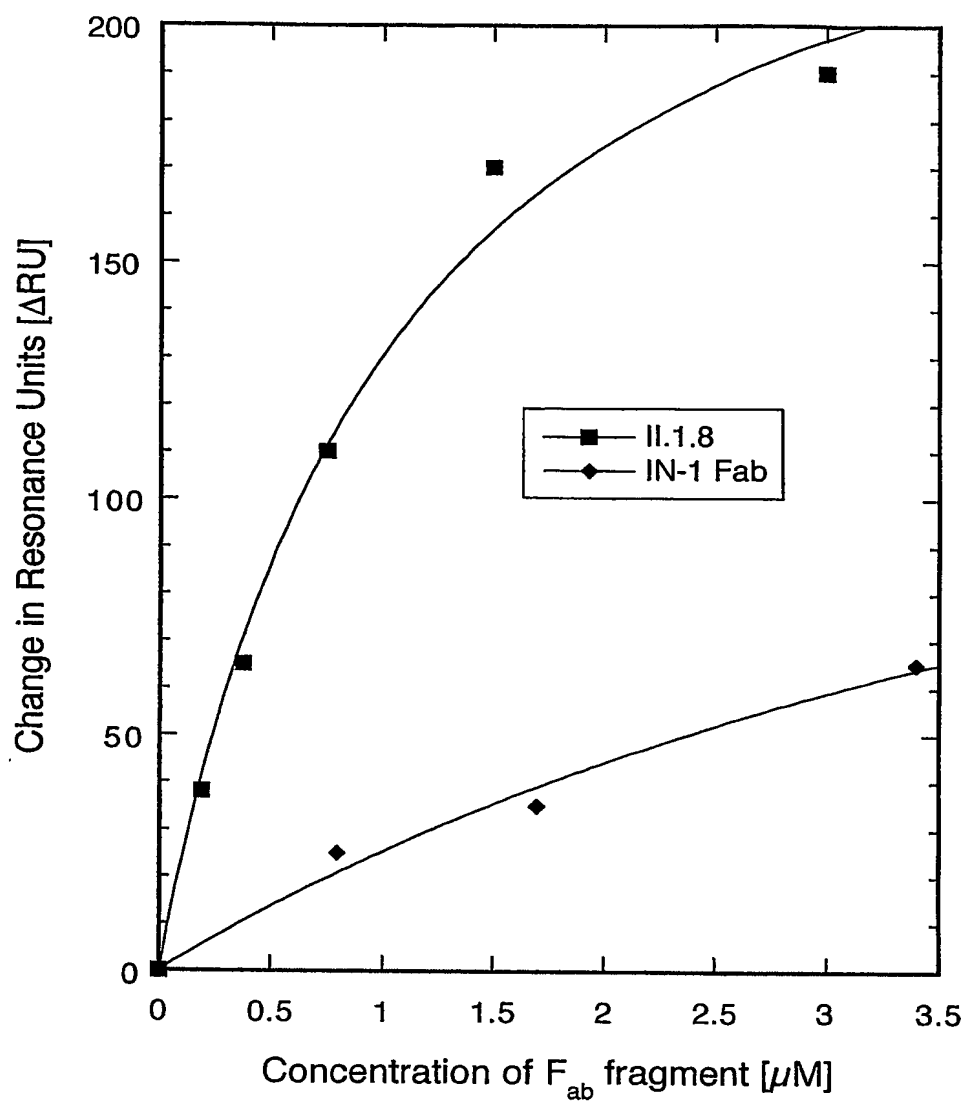


Fig. 3B



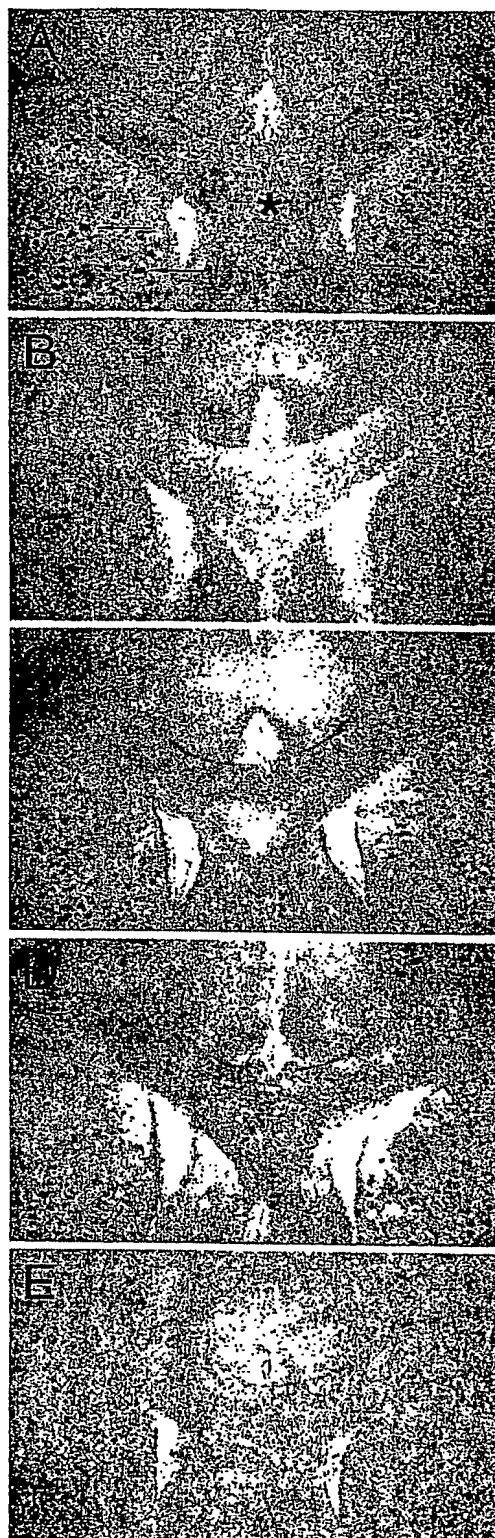


Fig. 4

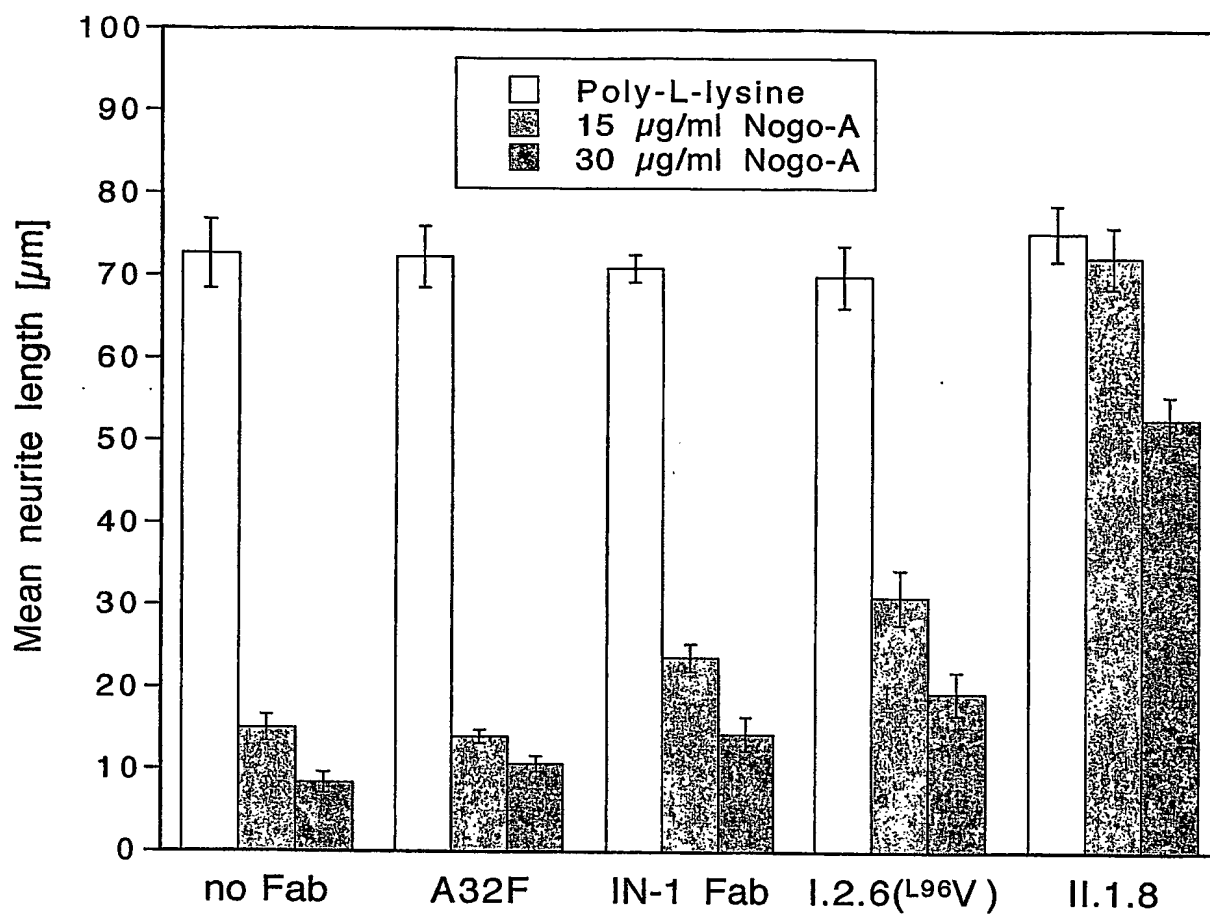


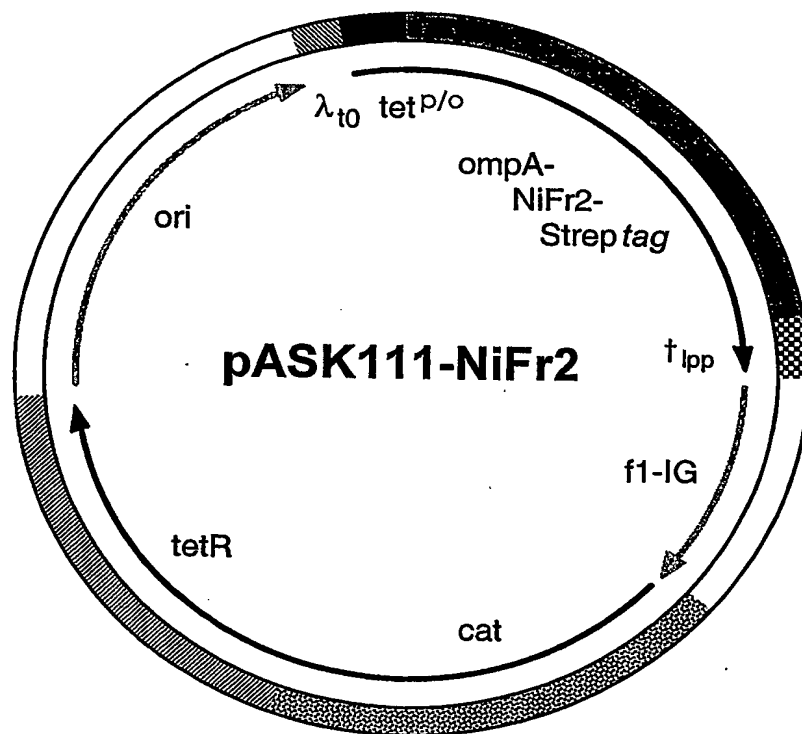
Fig. 5

MEDIDQSSLV	SSSTDSPPRP	PPAFKYQFVT	EPEDDEDEEEE	EEDEEEDDED	
LEELEVLERK	PAAGLSAAAV	PPAAAAPLLD	FSSDSVPPAP	RGPLPAAPPA	100
APERQPSWER	SPAAPAPSLP	PAAAVLPSKL	PEDDEPPARP	PPPPAGASP	
LAEPAAPST	PAAPKRRGSG	SVDETLFALP	AASEPVIPSS	AEKIMDLMEQ	200
PGNTVSSGQE	DFPSVLLETA	ASLPSLSPLS	TVSFKEHGYL	GNLSAVSSSE	
GTIEETLNEA	SKELPERATN	PFVNRDLAEF	SELEYSEMGS	SFKGSPKGES	300
AILVENTKEE	VIVRSKDKED	LVCSAALHSP	QESPVGKEDR	VVSPEKTMDI	
FNEMQMSVVA	PVREEYADFK	PFEQAWEVKD	TYEGSRDVL	ARANVESKVD	400
RKCLEDSLEQ	KSLGKDSEGR	NEDASFPSTP	EPVKDSSRAY	ITCASFTSAT	
ESTTANTFPL	LEDHTSENKT	DEKKIEERKA	QIITEKTSPK	TSNPFLVAVQ	500
DSEADYVTTD	TLSKVTEAAV	SNMPEGLTPD	LVQEACESEL	NEATGTKIAY	
ETKVDLVQTS	EAIQESLYPT	AQLCPSFEEA	EATPSPVLPD	IVMEAPLNSL	600
LPSAGASVVQ	PSVSPLEAPP	PVSYDSIKLE	PENPPPYEEA	MNVALKALGT	
KEGIKEPESF	NAAVQETEAP	YISIACDLIK	ETKLSTEPSP	DFSNYSEIAK	700
FEKSVPEHAE	LVEDSSPESE	PVDLFSDDSI	PEVPQTQEEA	VMLMKESLTE	
VSETVAQHKE	ERLSASPQEL	GKPYLESFQP	NLHSTKDAAS	NDIPTLTKKK	800
KISLQMEEFN	TAIYSNDDL	SSKEDKIKES	ETFSOSSPIE	IIDEFPTFVS	
AKDDSPKLAK	EYTDLEVSDK	SEIANIQSGA	DSLPCLELPC	DLSFKNIYPK	900
DEVHVSDEFS	ENRSSVSKAS	ISPSNVSALE	PQTEMGSIVK	SKSLTKEAEK	
KLPSDTEKED	RSLSAVLSAE	LSKTSVVDLL	YWRDIKKTGV	VFGASLELLL	1000
SLTVFSIVSV	TAYIALALLS	VTISFRIYKG	VIQAIQKSDE	GHPFRAYLES	
EVAISEELVQ	KYSNSALGHV	NSTIKELRRL	FLVDDLVDLS	KFAVLMWVFT	1100
YVGALFNGLT	LLILALISLF	SIPVIYERHQ	VQIDHYLGLA	NKSVKDAMAK	
IQAKIPGLKR	KAD				1163

Fig. 6A

MEDLDQSPLV	SSSDSPPRPQ	PAFKYQFVRE	PEDEEEEEEEE	EEDEDEDLE	
ELEVLERKPA	AGLSAAPVPT	APAAGAPLMD	FGNDFVPPAP	RGPLPAAPPV	100
APERQPSWDP	SPVSSTVPAP	SPLSAAAVSP	SKLPEDDEPP	ARPPPPPPAS	
VSPQAEPVWT	PPAPAPAAP	STPAAPKRRG	SSGSVDETLF	ALPAASEPVI	200
RSSAENMDLK	EQPGNTISAG	QEDFPSVLE	TAASLPSLSP	LSAASFKEHE	
YLGNLSTVLP	TEGTLQENV	EASKEVSEKA	KTLIDRDLT	EFSELEYSEM	300
GSSFVSFPA	ESAVIVANPR	EEIIVKNKDE	EEKLVSNIL	HNQQELPTAL	
TKLVKEDEVV	SSEKAKDSFN	EKRVAVEAPM	REEYADFKPF	ERVVEVKDSK	400
EDSDMLAAGG	KIESNLESKV	DKKCFADSLE	QTNHEKDSSES	SNDDTSFPST	
PEGIKDRPGA	YITCAPFNPA	ATESIATNIF	PLLGDPTSEN	KTDEKKIEEK	500
KAQIVTEKNT	STKTSNPFLV	AAQDSETDYV	TTDNLTQVTE	EVVANMPEGL	
TPDLVQEACE	SELNEVTGTK	IAYETKMDLV	QTSEVMQESL	YPAAQLCPSF	600
EESEATPSPV	LPDIVMEAPL	NSAVPSAGAS	VIQPSSSPLE	ASSVNYESIK	
HEPENPPPYE	EAMSVSLKKV	SGIKEEIKP	ENINAALQET	EAPYISIACD	700
LIKETKLSAE	PAPDFSDYSE	MAKVEQVPD	HSELVEDSSP	DSEPVDLFSD	
DSIPDVPQKQ	DETVMLVKES	LTETSFESMI	EYENKEKLSA	LPPEGGKPYL	800
ESFKLSLDNT	KDTLLPDEV	TLKKKEKIPL	QMEELSTAVY	SNDDLFIKE	
AQIRETETFS	DSSPIEIDE	FPTLISSKTD	SFSKLAREYT	DLEVSHKSEI	900
ANAPDGAGSL	PCTELPHDLS	LKNIQPKVEE	KISFSDDFSK	NGSATSKVLL	
LPPDVSAALAT	QAEIESIVKP	KVLVKEAEKK	LPSDTEKEDR	SPSAIFSAEL	1000
SKTSVVDLLY	WRDIKKTGVV	FGASLFLLLS	LTVFSIVSVT	AYIALALLSV	
TISFRIYKGV	IQAIQKSDEG	HPFRAYLESE	VAISEELVQK	YSNSALGHVN	1100
CTIKELRRLF	LVDDLVDLSK	FAVLMWVFTY	VGALFNGLTL	LILALISLFS	
VPVIYERHQA	QIDHYLGLAN	KNVKDAMAKI	QAKIPGLKRR	AE	1192

Fig. 6B



**Fig. 7**

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